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A comparative study on edible *Agaricus* mushrooms as functional foods

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Agaricus bisporus is a cultivated mushroom; *A. bitorquis*, *A. campestris* and *A. macrosporus* are edible mushrooms growing wild in nature. A chemical characterization was carried out with samples that originated in Serbia. Antioxidant, antimicrobial and anti-quorum sensing properties of their methanolic and ethanolic extracts were assessed. *A. campestris* had the lowest caloric value and total sugar content and showed the highest concentration in organic and phenolic acids, as also in tocopherols (mainly γ-tocopherol). In general, the methanolic extracts showed higher antioxidant, but lower antibacterial and antifungal potential than ethanolic ones. Sub-inhibitory concentrations of the ethanolic extracts demonstrated reduction of virulence factors, AQ inhibition zones, twitching and swimming motility. The biofilm forming capability of *P. aeruginosa* PAO1 was also reduced in a concentration-dependent manner at sub-MIC values. The extracts of the tested *Agaricus* species are a promising source of antioxidant, antimicrobial and antiquorum sensing compounds.

1. Introduction

The consumption of wild-growing mushrooms has been preferred to cultivated species in many countries of Europe. About 200 edible species have been identified in various parts of the world.[1] Important edible mushrooms belong to the *Agaricus* genus. *A. bisporus* is one of the most economically important edible species and, besides its nutritional value, it is also recognized for medicinal properties including antitumor, anti-aromatase, antimicrobial, immunomodulatory, anti-inflammatory as well as antioxidant.[2–6] *A. bitorquis* is an edible white mushroom, similar to the common button mushroom that is sold commercially. It is also commonly known as torq, banded agaric, spring agaric, or pavement mushroom, as it has been recorded pushing up paving slabs.[7] The meadow mushroom, *A. campestris*, is a white mushroom that is closely related to *A. bisporus*. In most areas it is a fall mushroom and, as its common and Latin names suggest, it comes up in meadows, fields, and grassy areas, after rains, and has a pleasant taste. *A. macrosporus* is known as Horse mushroom and is a stately and impressive species, recognized by its preference for grassy areas and sweetish smell.[7]

All these species are easy to recognize and they can be collected in large quantities. The taste and size of their fruiting bodies are important factors for considering these mushrooms as potential important foodstuffs. Although the wild edible mushrooms are commercialized at higher prices than the cultivated species, the majority of the consumers prefer wild mushrooms due to their characteristic flavor and texture. There are many reports on the nutritional value of cultivated and wild edible mushrooms from different countries, but no information is available regarding these three wild species from Serbia.

Antioxidants play an important role in defending the body against free radical attack by delaying or inhibiting the oxidation of lipids, DNA or proteins, preventing or repairing the damage to cells.[3,8] Furthermore, although the use of antimicrobial agents has been decreasing, the spread and severity of a wide variety of infectious diseases, as also the resistance developed by bacteria and fungi demands new alternatives.[9] Otherwise, many food products are perishable and also require protection from microbial spoilage during preparation, storage and distribution, in order to guarantee the acceptable shelf-life and organoleptic characteristics.

With these concepts in mind, the main focus of this study was to perform the chemical characterization of four *Agaricus* spp. from Serbia, regarding their nutritional value, hydrophilic and lipophilic compounds, and also to evaluate their biological activity (antioxidant, antimicrobial and antiquorum properties).

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2. Materials and methods

2.1. Mushroom species

Cultivated *A. bisporus* was bought at a local market (Belgrade, Serbia), and wild growing *A. bitorquis, A. campestris* and *A. macrosporus* (Avala mountain, Krupanj, Divcibare mountain, respectively) were collected in Serbia, in autumn 2013, and authenticated by Dr Jasmina Glamočlija (Institute for Biological Research, University of Belgrade, Serbia). 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 numbers Abis 12-2013, Abit 45-2013, Acam 23-2013, Amac 33-2013. All the samples were lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh), mixed to obtain homogeneous samples and stored in a desiccator, protected from light, until further analysis.

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 acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), and so were other individual fatty acid isomers and standards of tocopherols, ergosterol, sugars, organic acids and phenolic compounds, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Racemic tocol, 50 mg mL^{-1}, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethylsulfoxide (DMSO) (Merck KGaA, Germany) was used as a solvent. Phosphate buffered saline (PBS) was obtained from Sigma Chemical Co. (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, Greenville, SC, USA).

2.3. Chemical characterization of *Agaricus* spp.

(a) Nutritional value. The samples were analysed for their chemical composition (moisture, proteins, fat, carbohydrates and ash) through AOAC procedures.[10] 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 Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. Total carbohydrates were calculated by difference. The energy contribution is calculated according to the following equation: energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

(b) Hydrophilic compounds. *Sugars*. Following the extraction procedure described by Reis *et al.*[11] free sugars were determined by a High Performance Liquid Chromatography (HPLC) system consisting of an integrated system with a pump (Knauer, Smartline System 1000, Berlin, Germany), a degasser system (Smartline Manager 5000) and an auto-sampler (AS-2057 Jasco, Easton, MD, USA), coupled to a refraction index detector (RI detector Knauer Smartline 2300). Sugar identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). 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 the commercial standards of each compound. The results are expressed in g per 100 g of dry weight.

Organic acids. Following the extraction procedure described by Barros *et al.*[12] organic acids were determined by ultra fast liquid chromatography (UFLC, Shimadzu 20A Series, Kyoto, Japan) coupled with a photodiode array detector (PDA). The organic acids were quantified by the 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 g per 100 g of dry weight.

Phenolic compounds. Following the extraction procedure described by Barros *et al.*[13] phenolic acids and related compounds were determined using the UFLC mentioned above. Detection was carried out in a photodiode array detector (PDA), using 280 nm as the preferred wavelength. The phenolic acids and related compounds were quantified by a comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The results are expressed in mg per 100 g of dry weight.

(c) Lipophilic compounds. *Fatty acids*. Following the extraction transesterification procedures described by Reis *et al.*[11] fatty acids were determined using a gas chromatographer (DANI 1000, Contone, Switzerland) 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, Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

Tocopherols. Following the extraction procedure described by Heleno *et al.*[14] tocopherols were determined by HPLC (equipment described above, for sugar composition), and a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparison 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 are expressed in μg per 100 g of dry weight.

2.4. Extract preparation

The lyophilized powder (1 g) was extracted by stirring with 40 mL of methanol (25 °C, at 150 rpm) for 1 h and subsequently filtered through Whatman No. The residue was then

extracted with 20 mL of methanol for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210) to dryness. The ethanolic extracts were prepared following the procedure described by Cheng *et al.*[2] with some modifications. The dry fruiting bodies (1 g) were extracted by stirring with 30 mL of 90% ethanol during 48 h at 70 °C. The extracts were filtered and centrifuged to get a clear liquid, and evaporated at 40 °C. The extracts were re-dissolved in (a) the corresponding extraction solvent for the antioxidant activity assays (20 mg mL^{-1}), (b) 5% solution of DMSO in distilled water for the antimicrobial activity assays (100 mg mL^{-1}).

2.5. Evaluation of the antioxidant potential of the *Agaricus* spp. extracts

(a) General. Successive dilutions were made from the stock solution and subjected to different *in vitro* assays to evaluate the antioxidant activity of the samples.[15] The sample concentrations 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 from absorbance at 690 nm (ferricyanide/Prussian blue assay) against sample concentrations. Trolox was used as the standard.

(b) Folin–Ciocalteu assay. The extract solution (1 mL) was mixed with Folin–Ciocalteu reagent (5 mL, previously diluted with water 1 : 10, v/v) and sodium carbonate (75 g L^{-1}, 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 (Analytik Jena 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.

(c) 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^{-1}, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 well plates, the same with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm using a ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

(d) DPPH radical-scavenging activity assay. This methodology was performed using the Microplate Reader mentioned above. The reaction mixture was prepared in a 96 well plates and consisted of 30 μL of a concentration range of the extract and 270 μL methanol containing DPPH radicals (6×10^{-5} mol L^{-1}). 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: % 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.

(e) Inhibition of the 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 40 °C 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 0.2 mL of a concentration range of the extract. The tubes were shaken and incubated at 50 °C 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 2 h of assay/initial absorbance) × 100.

(f) 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* for 10 min. An aliquot (100 μL) of the supernatant was incubated with 200 μL samples of a concentration range of the extract in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL) at 37 °C 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 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color 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 (%) = $[(A - B)/A] \times 100\%$, where *A* and *B* are the absorbance of the control and the sample solution, respectively.

2.6. Evaluation of the antimicrobial activity of the *Agaricus* spp. extracts

(a) Antibacterial activity. The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240) and *Listeria monocytogenes* (NCTC 7973), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 35210), and *Enterobacter cloacae* (human isolate), were used. The antibacterial assay was carried out by a microdilution method.[16,17] The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU mL^{-1}. Mushroom extracts were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg mL^{-1}) and added in Tryptic Soy broth (TSB) medium (100 μL) with bacterial inoculum (1.0×10^4 CFU per well). The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MICs obtained from the susceptibility testing of various bacteria to tested extracts were also determined by a colorimetric microbial viability assay based on reduction of an INT ((*p*-iodonitrotetrazolium violet) [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; Sigma]) color and compared with positive control for each bacterial strain. The MBCs were determined by serial sub-cultivation of 2 μL of tested compounds into microtitre plates con-

taining 100 μL of broth per well and further incubated for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by using Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank (broth medium plus diluted extracts) and the positive control. Streptomycin (Sigma P 7794) and ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg mL^{-1} in sterile physiological saline). Five percent DMSO was used as a negative control.

(b) Antifungal activity. *Aspergillus fumigatus* (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (food isolate) were used. In order to investigate the antifungal activity of mushroom extract, a modified microdilution technique was used.[18] The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v) and spore suspension was adjusted with sterile saline to a concentration of 1.0×10^5. Extracts were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg mL^{-1}) and added in a broth malt medium with an inoculum (0.005–3 mg mL^{-1} for extracts). The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 μL of tested compounds dissolved in medium and incubated for 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, and commercial fungicides, bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia), were used as positive controls (1–3000 μg mL^{-1}). Five percent DMSO was used as a negative control.

2.7. Antiquorum sensing (AQ) activity of mushroom extracts

(a) Bacterial strains, growth media and culture conditions. *P. aeruginosa PA01* (ATCC 27853) used in this study was from the collection of the Mycoteca, Institute for Biological Research "Sinisa Stankovic", Belgrade, Serbia. Bacteria were routinely grown in Luria–Bertani (LB) medium (1% w/v NaCl, 1% w/v Tryptone, 0.5% w/v yeast extract) with shaking (220 rpm) and cultured at 37 °C.

(b) Biofilm formation. The effect of different concentrations of extracts (ranging from 0.5, 0.25 and 0.125 of MIC) on biofilm forming ability was tested on polystyrene flat-bottomed microtitre 96 well plates as described by Drenkard & Ausubel[19] with some modifications. Briefly, 100 μL of an overnight culture of *P. aeruginosa* (inoculum size was 1×10^8 CFU mL^{-1}) was added to each well of the plates in the presence of 100 μL subinhibitory concentrations (subMIC) of extracts (0.5, 0.25 and 0.125 MIC) or 100 mL medium (control). After incubation for 24 h at 37 °C, each well was washed twice with sterile PBS (pH 7.4), dried, and stained for 10 min with 0.1% crystal violet in order to determine the biofilm mass. After drying, 200 μL of 95% ethanol (v/v) was added to solubilize the dye that had stained the biofilm cells. The excess stain was washed off with dH_2O. After 10 min, the content of the wells was homogenized and the absorbance at λ = 625 nm was read on a Sunrise™ Tecan ELISA reader. The experiment was done in triplicate and repeated two times and values were presented as mean values ± SE.

(c) Discs-diffusion method for determination of AQ activity of mushroom extracts against *P. aeruginosa*. *P. aeruginosa* was cultured overnight at 37 °C in LB medium and then adjusted to a concentration of 1.0×10^8 CFU mL^{-1} for final inoculum. Filter paper discs (Whatman; 4 mm in diameter) were impregnated with a solution of *Agaricus* spp. extracts (2.50, 1.25, 0.60, 0.30, 0.15 mg per disc), streptomycin and ampicillin (2.50, 1.25, 0.60, 0.30, 0.15 mg per disc). Discs were dried at room temperature (3 h, protected from light), and aseptically placed onto the plates prior inoculated with *P. aeruginosa* (1×10^8 CFU mL^{-1}). Petri dishes then were placed for incubation in a thermostat at 37 °C for 24 h. After incubation, it was recorded whether inhibition or antiquorum zones were obtained. Minimal inhibitory concentrations were determined as the diameter of the growth clear inhibition zones around the discs (no growth), while antiquorum zones were determined as the transparent zones around the discs behind the margin of the inhibition zone.[20]

(d) Twitching and flagella motility. After growth in the presence or absence of *Agaricus* spp. extracts (subMIC), streptomycin and ampicillin (subMIC), the cells of *P. aeruginosa* PA01 were washed twice with sterile PBS and resuspended in PBS at 1×10^8 cfu mL^{-1} (OD of 0.1 at 660 nm). Briefly, cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37 °C. Plates were then removed from the incubator and incubated at room temperature for two more days. Colony edges and the zone of motility were measured with a light microscope.[21] Fifty microlitres of extracts was mixed into 10 mL of molten MH medium and poured immediately over the surface of a solidified LBA plate as an overlay. The plate was point inoculated with an overnight culture of PAO1 once the overlaid agar had solidified and incubated at 37 °C for 3 days. The extent of swimming was determined by measuring the area of the colony.[22] The experiment was done in triplicate and repeated two times.

2.8. Statistical analysis

For each species, three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard errors, and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with α = 0.05. This analysis was carried out using the SPSS v. 22.0 program.

3. Results and discussion

3.1. Chemical characterization of *Agaricus* spp.

The results of the chemical characterization of four *Agaricus* species from Serbia are shown in Tables 1–3. Carbohydrates

Table 1 Nutritional value and hydrophilic compounds in the studied *Agaricus* spp. (mean ± SD)[a]

Nutritional value (g per 100 g dw)	Ash	Proteins	Fat	Carbohydrates	Energy (kcal per 100 g dw)
Agaricus bisporus	15.02 ± 0.33^{b}	10.00 ± 0.37^{d}	3.12 ± 0.01^{ab}	71.86 ± 0.52^{a}	355.51 ± 0.95^{c}
Agaricus bitorquis	13.79 ± 0.16^{c}	24.88 ± 1.45^{a}	3.22 ± 0.07^{a}	58.11 ± 1.38^{c}	360.94 ± 0.21^{b}
Agaricus campestris	17.65 ± 0.25^{a}	19.12 ± 0.17^{c}	3.02 ± 0.07^{b}	60.21 ± 0.34^{c}	344.54 ± 0.46^{d}
Agaricus macrosporus	10.41 ± 0.43^{d}	21.87 ± 1.40^{b}	2.35 ± 0.07^{c}	65.37 ± 1.25^{b}	370.12 ± 0.97^{a}

Sugars (g per 100 g dw)	Fructose	Mannitol	Sucrose	Trehalose	Total
Agaricus bisporus	Nd	11.31 ± 0.09^{a}	nd	0.60 ± 0.06^{c}	11.91 ± 0.03^{a}
Agaricus bitorquis	0.40 ± 0.01^{b}	7.04 ± 0.40^{b}	1.49 ± 0.03	2.27 ± 0.02^{a}	11.20 ± 0.41^{b}
Agaricus campestris	0.29 ± 0.01^{b}	5.59 ± 0.18^{c}	nd	0.63 ± 0.05^{c}	6.51 ± 0.14^{d}
Agaricus macrosporus	2.65 ± 0.05^{a}	4.98 ± 0.07^{d}	nd	1.15 ± 0.02^{b}	8.78 ± 0.13^{c}

Organic acids (g per 100 g dw)	Oxalic acid	Quinic acid	Malic acid	Citric acid	Fumaric acid	Total
Agaricus bisporus	3.73 ± 0.03^{ab}	nd	3.82 ± 0.28^{b}	nd	0.28 ± 0.00^{b}	7.83 ± 0.31^{b}
Agaricus bitorquis	4.05 ± 0.17^{a}	nd	4.40 ± 0.21^{a}	nd	0.23 ± 0.00^{bc}	8.68 ± 0.38^{b}
Agaricus campestris	3.47 ± 0.36^{b}	nd	4.44 ± 0.19^{a}	2.39 ± 0.16^{a}	0.65 ± 0.01^{a}	10.95 ± 0.72^{a}
Agaricus macrosporus	0.26 ± 0.01^{c}	2.59 ± 0.32	1.74 ± 0.15^{c}	0.36 ± 0.04^{b}	0.20 ± 0.00^{c}	5.14 ± 0.51^{c}

Phenolic compounds (mg per 100 g dw)	Gallic acid	Protocatechuic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid	Total phenolic acids	Cinnamic acid
Agaricus bisporus	0.32 ± 0.00	nd	nd	0.12 ± 0.00^{b}	0.44 ± 0.00^{b}	0.07 ± 0.00^{b}
Agaricus bitorquis	nd	nd	0.03 ± 0.01^{b}	nd	0.03 ± 0.01^{c}	0.08 ± 0.00^{b}
Agaricus campestris	nd	1.07 ± 0.02	4.13 ± 0.12^{a}	0.68 ± 0.00^{a}	5.88 ± 0.10^{a}	1.75 ± 0.02^{a}
Agaricus macrosporus	nd	nd	nd	nd	nd	0.08 ± 0.00^{b}

[a] nd, not detected; dw, dry weight. In each column different letters mean significant differences between species ($p < 0.05$).

Table 2 Lipophilic compounds in the studied *Agaricus* spp. (mean ± SD)[a]

Fatty acids (percentage)	*Agaricus bisporus*	*Agaricus bitorquis*	*Agaricus campestris*	*Agaricus macrosporus*
C6:0	0.06 ± 0.00	0.03 ± 0.00	0.11 ± 0.02	0.05 ± 0.01
C8:0	0.04 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.02 ± 0.00
C10:0	0.04 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
C12:0	0.09 ± 0.00	0.06 ± 0.01	0.11 ± 0.01	0.03 ± 0.01
C13:0	nd	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
C14:0	0.61 ± 0.01	0.66 ± 0.06	0.78 ± 0.01	0.28 ± 0.02
C14:1	0.01 ± 0.00	nd	0.02 ± 0.00	nd
C15:0	0.32 ± 0.00	0.28 ± 0.06	0.51 ± 0.00	0.59 ± 0.03
C15:1	nd	nd	nd	nd
C16:0	15.40 ± 0.20	12.69 ± 0.18	13.17 ± 0.16	10.88 ± 0.25
C16:1	0.23 ± 0.01	0.28 ± 0.02	1.62 ± 0.02	0.23 ± 0.03
C17:0	0.38 ± 0.01	0.44 ± 0.05	0.76 ± 0.01	0.98 ± 0.04
C18:0	3.71 ± 0.03	4.99 ± 0.36	3.51 ± 0.03	3.08 ± 0.00
C18:1n9	14.91 ± 0.02	5.47 ± 0.15	3.52 ± 0.05	2.62 ± 0.06
C18:2n6	60.36 ± 0.25	69.86 ± 1.48	71.40 ± 0.09	74.90 ± 0.06
C18:3n6	nd	±	±	0.67 ± 0.03
C18:3n3	0.89 ± 0.01	0.92 ± 0.11	0.19 ± 0.02	0.16 ± 0.03
C20:0	1.17 ± 0.02	1.49 ± 0.14	1.36 ± 0.00	1.46 ± 0.04
C20:1	0.16 ± 0.01	0.21 ± 0.05	0.13 ± 0.00	0.02 ± 0.00
C20:2	0.11 ± 0.02	0.09 ± 0.02	0.23 ± 0.00	0.10 ± 0.01
C20:3n3 + C21:0	0.17 ± 0.00	0.14 ± 0.01	0.23 ± 0.01	0.40 ± 0.02
C20:5n3	0.08 ± 0.01	nd	0.08 ± 0.01	0.11 ± 0.02
C22:0	0.73 ± 0.01	1.43 ± 0.26	1.20 ± 0.00	2.30 ± 0.08
C22:1n9	nd	nd	0.01 ± 0.00	0.03 ± 0.00
C23:0	0.08 ± 0.03	0.17 ± 0.03	0.18 ± 0.00	0.22 ± 0.01
C24:0	0.46 ± 0.02	0.74 ± 0.18	0.80 ± 0.01	0.86 ± 0.02
C24:1	nd	nd	nd	0.02 ± 0.00
Total SFA (% of total FA)	23.08 ± 0.26a	23.03 ± 1.19a	22.57 ± 0.19b	20.76 ± 0.14c
Total MUFA (% of total FA)	15.31 ± 0.02a	5.97 ± 0.19b	5.30 ± 0.07c	2.91 ± 0.10d
Total PUFA (% of total FA)	61.61 ± 0.24d	71.01 ± 1.40c	72.13 ± 0.12b	76.33 ± 0.04a
Tocopherols (µg per 100 g dw)				
α-Tocopherol	nd	5.14 ± 0.40^{ab}	6.36 ± 0.40^{a}	4.08 ± 1.28^{c}
β-Tocopherol	25.26 ± 0.30	nd	nd	nd
γ-Tocopherol	nd	10.97 ± 0.49^{c}	109.83 ± 1.39^{a}	26.88 ± 4.67^{b}
δ-Tocopherol	nd	18.79 ± 1.38	nd	nd
Total tocopherols	25.26 ± 0.30^{c}	34.90 ± 1.49^{b}	116.19 ± 1.79^{a}	30.96 ± 3.39^{b}

[a] nd, not detected; dw, dry weight. In each line different letters mean significant differences between species ($p < 0.05$).

Table 3 Antioxidant properties of the methanolic (MeOH) and ethanolic (EtOH) extracts of the studied *Agaricus* spp. (mean ± SD)[a]

	Folin–Ciocalteu assay (mg GAE per g extract)	Ferricyanide/Prussian blue assay (EC_{50}; mg mL^{-1})	DPPH radical-scavenging activity assay (EC_{50}; mg mL^{-1})	β-Carotene/linoleate assay (EC_{50}; mg mL^{-1})	TBARS assay (EC_{50}; mg mL^{-1})
MeOH					
Agaricus bisporus	35.35 ± 0.24^{c}	1.37 ± 0.02^{b}	3.72 ± 0.06^{b}	3.18 ± 0.21^{c}	0.59 ± 0.06^{b}
Agaricus bitorquis	127.19 ± 1.24^{a}	0.74 ± 0.02^{c}	3.44 ± 0.10^{c}	3.36 ± 0.13^{b}	1.46 ± 0.23^{a}
Agaricus campestris	48.19 ± 0.16^{b}	0.72 ± 0.01^{c}	1.18 ± 0.05^{d}	0.28 ± 0.03^{d}	0.04 ± 0.01^{c}
Agaricus macrosporus	24.27 ± 0.50^{d}	1.75 ± 0.04^{a}	6.15± 0.25^{a}	4.17 ± 0.13^{a}	1.47 ± 0.11^{a}
EtOH					
Agaricus bisporus	11.33 ± 0.29^{c}	8.07 ± 0.19^{b}	20.12 ± 0.55^{b}	16.99 ± 0.40^{b}	13.76 ± 0.02^{a}
Agaricus bitorquis	139.25 ± 0.19^{a}	1.30 ± 0.01^{c}	2.41 ± 0.09^{c}	1.29 ± 0.20^{c}	2.50 ± 0.08^{c}
Agaricus campestris	56.79 ± 1.58^{b}	0.88 ± 0.02^{d}	0.64 ± 0.02^{d}	0.48 ± 0.02^{d}	0.82 ± 0.62^{d}
Agaricus macrosporus	11.78 ± 0.12^{c}	9.86 ± 0.15^{a}	36.05 ± 0.89^{a}	17.97 ± 1.48^{a}	4.71 ± 0.47^{b}

[a] In each line different letters mean significant differences between species ($p < 0.05$). Concerning the Folin–Ciocalteu assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, which means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: the extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the ferricyanide/Prussian blue assay. GAE, gallic acid equivalents.

were the most abundant macronutrients present in the studied mushrooms (58–72 g per 100 g dw; Table 1), *A. bisporus* being the most rich species in these macronutrients. Ash (10–15 g per 100 g dw) and fat (2–3 g per 100 g dw) contents were low and their energy contribution (344–370 kcal per 100 g dw) was mainly due to carbohydrates and proteins (11–25 g per 100 g

dw). The main sugars were mannitol (5–11 g per 100 g dw), trehalose (0.6–2 g per 100 g dw) and fructose (0.3–3 g per 100 g dw), while sucrose was detected only in *A. bitorquis* (1.5 g per 100 g dw; Table 1). Regarding organic acids, the main molecules found in the studied species were malic (3–4 g per 100 g dw) and oxalic (0.3–4 g per 100 g dw) acids; fumaric acid was detected in lower amounts (0.2–0.6 g per 100 g dw), while citric acid was observed only in *A. campestris* (2.4 g per 100 g dw) and in *A. macrosporus* (0.4 g per 100 g dw), and malic acid in *A. macrosporus* (2.6 g per 100 g dw) (Table 1). The phenolic acids found in the present study were gallic, protocatechuic, *p*-hydroxybenzoic and *p*-coumaric acids, as also the related compound cinnamic acid (Table 1). Gallic acid was only found in *A. bisporus* (0.3 mg per 100 g dw), protocatechuic (1.1 mg per 100 g dw) and *p*-hydroxybenzoic (4.1 mg per 100 g dw) acids in *A. macrosporus*, and *p*-coumaric acid in *A. bisporus* (0.1 mg per 100 g dw) and *A. macrosporus* (0.7 mg per 100 g dw); cinnamic acid was quantified in all the species (0.1–1.8 mg per 100 g dw) (Table 1).

Concerning the fatty acid composition of the studied species (Table 2), polyunsaturated fatty acids (PUFA, 62–76% of total fatty acids (FA)) predominated over saturated fatty acids (SFA, 21–23% of total FA) and monounsaturated fatty acids (MUFA, 3–15% of total FA). *A. bisporus* and *A. bitorquis* presented the highest content in SFA, the first species also in MUFA, while *A. macrosporus* gave the highest percentage of PUFA (Table 2). Thus, all the species seem to be excellent options as food. The fatty acids found in higher amounts were palmitic acid (C16:0, SFA); oleic acid (C18:1n9, MUFA), and linoleic acid (C18:2n6, PUFA). Similar profiles were detected in cultivated species from Portugal.[11] Regarding tocopherols, α-, β-, γ- and δ- isoforms were quantified (Table 2). β-Tocopherol was only found in *A. bisporus* (25 μg per 100 g dw), while δ-tocopherol was detected in *A. bitorquis* (18 μg per 100 g dw). Total tocopherols were presented in higher concentration (116 μg per 100 g dw) in *A. campestris* due to the contribution of γ-tocopherol (110 μg per 100 g dw) (Table 2).

3.2. Antioxidant and antimicrobial activities of *Agaricus* spp. extracts

Antioxidant activity of the investigated *Agaricus* species is presented in Table 3. Both methanolic and ethanolic extracts of the studied species have shown antioxidant potential, but the first one was better in most of the cases (Table 3). Methanolic and ethanolic extracts of *A. bitorquis* presented the highest total phenolic content (127 and 130 mg GAE per g extract, respectively). Among the methanolic extracts, *A. campestris* revealed the highest reducing power (EC_{50} = 0.7 mg mL^{-1}), DPPH scavenging activity (EC_{50} = 1.2 mg mL^{-1}), β-carotene bleaching inhibition (EC_{50} = 0.3 mg mL^{-1}) and TBARS formation decrease (EC_{50} = 0.04 mg mL^{-1}). The same tendency was observed for ethanolic extracts (EC_{50} = 0.9, 0.6, 0.5 and 0.8 mg mL^{-1}, respectively; Table 3). This species (*A. campestris*) was also the one that showed the highest phenolic acid concentration (Table 2). To date there are various antioxidant activity assays, each one having their specific target within the matrix and all of them with advantages and disadvantages. There is not one method that can provide unequivocal results and the best solution is to use various methods instead of a one-dimension approach. Some of these procedures use free radicals, some are specific for lipid peroxidation and tend to need animal or plant cells, some have a broader scope, some require minimum preparation and knowledge, few reagents and are quick to produce outputs. Thus, it is very important to use different antioxidant assays in order to get a better overview of the results and applicability of natural matrices such are mushrooms.

The results of antibacterial and antifungal activities of methanolic and ethanolic extracts of the tested *Agaricus* species are presented in Table 4. Ethanolic extracts of all the tested species exhibited higher antibacterial activity than methanolic ones, with exception towards *L. monocytogenes.* The best antibacterial effect was achieved by *A. macrosporus* extracts against all bacteria, except *L. monocytogenes. A. bitorquis* extracts showed the best effect against this bacterium. Extracts of *A. bisporus* possessed the lowest antibacterial activity among all the others. The antibacterial activity displayed by the extracts was lower than that demonstrated by the antibiotics.

Ethanolic extracts of all the tested species showed once more the highest antifungal activity, with few exceptions; *A. bisporus* ethanolic extract exhibited a lower effect than methanolic extracts towards *A. ochraceus* and *T. viride*, and *A. macrosporus* ethanolic extract possessed lower effect than methanolic extract against *P. funiculosum* and *P. ochrochloron.* The best antifungal activity was obtained for *A. macrosporus* extracts against all the tested microfungi. These extracts also showed higher or similar inhibitory activity than ketoconazole, and an even higher fungicidal effect against *P. funiculosum.* Extracts of *A. campestris* exhibited the worst antifungal potential among all the tested strains.

3.3. Antiquorum sensing activity of *Agaricus* spp. extracts

The effect of *Agaricus* spp. ethanolic extracts on the biofilm formation of *P. aeruginosa* was tested with 0.5, 0.25 and 0.125 of the determined MIC. Table 5 shows that all the tested extracts, at 0.5 MIC, reduced biofilm formation more than streptomycin and ampicillin. The extracts reduced biofilm formation in the range of 53–87%, which means that the biofilm was formed in the presence of extracts in the range of 13–47%. The best results were observed for the *A. macrosporus* extract, while *A. campestris* showed the lowest reduction of biofilm formation. Streptomycin and ampicillin reduced biofilm by 51% and 31%, respectively. Extracts tested at 0.25 MIC exhibited slightly higher reduction of biofilm production than positive controls, while at 0.125 MIC they possessed lower activity.

The quorum-sensing inhibition zones were determined by the disc diffusion method. It can be seen that the extracts of *A. bisporus* (8.0–15.0 mm) and *A. bitorquis* (7.0–8.7 mm) showed antiquorum sensing (AQ) activity at all concentrations. Ampicillin possessed AQ activity at higher concentration (7.6 mm), while streptomycin showed the best AQ activity presenting the zones in the range of 15.5–22.6 mm.

Table 4 Antimicrobial activity of the methanolic (MeOH) and ethanolic (EtOH) extracts of the studied *Agaricus* spp. (mean ± SD)[a]

		A. bisporus		*A. bitorquis*		*A. campestris*		*A. macrosporus*			
Bacteria		Me	Et	Me	Et	Me	Et	Me	Et	Strep	Ampic
S. aurues	MIC	0.035 ± 0.02^{a}	0.145 ± 0.002^{c}	2.345 ± 0.00^{e}	0.230 ± 0.00^{d}	2.345 ± 0.02^{e}	0.035 ± 0.002^{f}	0.450 ± 0.02^{d}	0.350 ± 0.00^{e}	0.250 ± 0.020^{c}	0.100 ± 0.007^{b}
	MBC	4.690 ± 0.06^{d}	4.690 ± 0.03^{e}	4.690 ± 0.03^{d}	0.940 ± 0.01^{d}	4.690 ± 0.06^{d}	4.690 ± 0.03^{e}	3.000 ± 0.00^{c}	0.580 ± 0.007^{c}	0.500 ± 0.007^{b}	0.150 ± 0.070^{a}
B. cereus	MIC	2.345 ± 0.002^{e}	0.035 ± 0.00^{a}	1.170 ± 0.01^{d}	0.840 ± 0.01^{c}	1.170 ± 0.02^{b}	0.072 ± 0.0007^{b}	0.450 ± 0.003^{c}	1.170 ± 0.07^{e}	0.050 ± 0.00^{a}	0.100 ± 0.007^{b}
	MBC	4.690 ± 0.06^{d}	4.690 ± 0.03^{d}	2.345 ± 0.02^{b}	0.940 ± 0.01^{b}	2.345 ± 0.02^{b}	2.345 ± 0.00^{c}	3.000 ± 0.07^{c}	2.300 ± 0.002^{c}	0.100 ± 0.30^{a}	0.150 ± 0.00^{a}
L. monocytogenes	MIC	0.290 ± 0.003^{b}	0.145 ± 0.02^{a}	0.290 ± 0.003^{b}	0.940 ± 0.10^{b}	0.580 ± 0.003^{d}	2.345 ± 0.20^{c}	0.400 ± 0.007^{c}	2.300 ± 0.07^{c}	0.150 ± 0.003^{a}	0.150 ± 0.00^{a}
	MBC	0.145 ± 0.02^{a}	9.370 ± 0.10^{d}	0.585 ± 0.002^{c}	1.870 ± 0.02^{b}	2.345 ± 0.02^{d}	4.690 ± 0.03^{c}	3.000 ± 0.07^{e}	4.600 ± 0.03^{c}	0.300 ± 0.00^{b}	0.300 ± 0.01^{b}
M. flavus	MIC	4.690 ± 0.04^{d}	0.145 ± 0.002^{a}	2.345 ± 0.02^{c}	1.170 ± 0.10^{b}	2.345 ± 0.09^{c}	0.145 ± 0.02^{a}	1.150 ± 0.05^{b}	1.170 ± 0.01^{b}	0.130 ± 0.01^{a}	0.100 ± 0.00^{a}
	MBC	9.370 ± 0.10^{d}	9.370 ± 0.10^{e}	4.690 ± 0.10^{c}	1.870 ± 0.10^{b}	4.690 ± 0.03^{c}	4.690 ± 0.03^{d}	3.000 ± 0.20^{b}	2.300 ± 0.07^{c}	0.250 ±0.007^{a}	0.150 ± 0.01^{a}
P. aeruginosa	MIC	2.345 ± 0.02^{c}	0.585 ± 0.03^{c}	2.345 ± 0.08^{c}	0.940 ± 0.01^{d}	2.345 ± 0.10^{c}	0.325 ± 0.008^{b}	0.750 ± 0.02^{b}	0.580 ± 0.03^{c}	0.050 ± 0.00^{a}	0.100 ± 0.00^{a}
	MBC	4.690 ± 0.20^{c}	9.370 ± 0.10^{e}	4.690 ± 0.10^{c}	1.870 ± 0.10^{c}	4.690 ± 0.06^{c}	4.690 ± 0.20^{d}	1.500 ± 0.05^{b}	1.170 ± 0.06^{b}	0.100 ± 0.00^{a}	0.200 ± 0.01^{a}
E. coli	MIC	4.690 ± 0.03^{e}	0.585 ± 0.03^{d}	2.345 ± 0.02^{d}	1.170 ± 0.01^{e}	2.345 ± 0.00^{d}	0.145 ± 0.00^{b}	0.750 ± 0.02^{c}	1.170 ± 0.002^{e}	0.050 ± 0.002^{a}	0.300 ± 0.01^{b}
	MBC	9.370 ± 0.10^{e}	9.370 ± 0.03^{d}	4.690 ± 0.20^{d}	1.870 ± 0.01^{e}	4.690 ± 0.00^{d}	4.690 ± 0.002^{b}	1.500 ± 0.003^{c}	4.600 ± 0.02^{e}	0.100 ± 0.007^{a}	0.500 ± 0.02^{b}
S. typhimurium	MIC	0.035 ± 0.002^{a}	0.145 ± 0.02^{bc}	1.170 ± 0.06^{b}	0.470 ± 0.02^{d}	2.345 ± 0.002^{a}	0.035 ± 0.006^{e}	0.750 ± 0.02^{c}	1.170 ± 0.07^{b}	0.050 ± 0.007^{a}	0.150 ± 0.007^{a}
	MBC	4.690 ± 0.03^{d}	4.690 ± 0.20^{d}	2.345 ± 0.10^{c}	1.870 ± 0.30^{b}	4.690 ± 0.20^{d}	2.345 ± 0.10^{c}	1.500 ± 0.07^{b}	2.300 ± 0.10^{c}	0.100 ± 0.007^{a}	0.200 ± 0.007^{a}
E. cloacae	MIC	0.072 ± 0.0007^{a}	0.145 ± 0.02^{b}	1.170 ± 0.06^{d}	0.230 ± 0.01^{c}	2.345 ± 0.02^{e}	0.035 ± 0.002^{a}	0.400 ± 0.02^{c}	1.170 ± 0.06^{d}	0.050 ± 0.00^{a}	0.150 ± 0.007^{b}
	MBC	4.690 ± 0.20^{d}	4.690 ± 0.20^{d}	2.345 ± 0.02^{c}	0.940 ± 0.01^{b}	4.690 ± 0.20^{d}	2.345 ± 0.10^{c}	0.750 ± 0.01^{b}	2.300 ± 0.10^{c}	0.100 ± 0.01^{a}	0.200 ± 0.01

		A. bisporus		*A. bitorquis*		*A. campestris*		*A. macrosporus*			
Fungi		Me	Et	Me	Et	Me	Et	Me	Et	Ketoc	Bifon
A. fumigatus	MIC	0.200 ± 0.01^{a}	1.560 ± 0.20^{b}	3.120 ± 0.04^{b}	3.120 ± 0.07^{d}	6.250 ± 0.08^{c}	3.120 ± 0.00^{d}	3.000 ± 0.07^{b}	2.340 ± 0.10^{c}	0.200 ± 0.01^{a}	0.150 ± 0.01^{a}
	MFC	12.500 ± 0.20^{d}	3.120 ± 0.04^{c}	6.250 ± 0.08^{c}	6.250 ± 0.08^{d}	22.500 ± 0.20^{e}	3.250 ± 0.08^{c}	6.000 ± 0.00^{c}	3.125 ± 0.04^{c}	0.500 ± 0.02^{b}	0.200 ± 0.00^{a}
A. versicolors	MIC	3.120 ± 0.04^{e}	3.120 ± 0.04^{d}	0.780 ± 0.01^{c}	0.390 ± 0.003^{b}	1.560 ± 0.02^{d}	0.390 ± 0.00^{c}	0.750 ± 0.003^{c}	1.560 ± 0.09^{c}	0.200 ± 0.003^{b}	0.100 ± 0.00^{a}
	MFC	6.250 ± 0.08^{e}	6.250 ± 0.08^{d}	3.120 ± 0.04^{d}	3.120 ± 0.04^{c}	12.500 ± 0.20^{f}	3.120 ± 0.007^{c}	1.500 ± 0.07^{c}	3.125 ± 0.08^{c}	0.500 ± 0.01^{b}	0.200 ± 0.007^{a}
A. ochraceus	MIC	0.560 ± 0.01^{b}	3.120 ± 0.04^{e}	0.780 ± 0.01^{c}	0.390 ± 0.003^{b}	1.560 ± 0.02^{d}	0.790 ± 0.003^{d}	1.500 ± 0.00^{e}	0.500 ± 0.01^{c}	0.150 ± 0.01^{a}	0.150 ± 0.00^{a}
	MFC	0.780 ± 0.03^{b}	6.250 ± 0.08^{d}	3.120 ± 0.04^{c}	3.120 ± 0.04^{c}	12.500 ± 0.20^{d}	3.120 ± 0.00^{c}	3.000 ± 0.10^{c}	0.780 ± 0.007^{b}	0.200 ± 0.01^{a}	0.200 ± 0.00^{a}
A. niger	MIC	0.560 ± 0.02^{b}	0.560 ± 0.02^{b}	3.120 ± 0.04^{c}	1.560 ± 0.00^{c}	6.250 ± 0.08^{d}	0.100 ± 0.01^{a}	3.000 ± 0.00^{c}	2.340 ± 0.10^{d}	0.200 ± 0.01^{a}	0.150 ± 0.007^{a}
	MFC	6.250 ± 0.08^{cd}	3.120 ± 0.04^{c}	6.250 ± 0.00^{d}	6.250 ± 0.08^{d}	12.500 ± 0.20^{e}	3.120 ± 0.00^{c}	6.000 ± 0.00^{c}	3.125 ± 0.002^{c}	0.500 ± 0.00^{b}	0.200 ± 0.01^{a}
T. viride	MIC	0.780 ± 0.07^{d}	3.120 ± 0.04^{e}	0.780 ± 0.007^{d}	0.780 ± 0.03^{d}	3.120 ± 0.04^{e}	0.390 ± 0.01^{c}	0.400 ± 0.20^{c}	0.780 ± 0.00^{d}	0.200 ± 0.01^{b}	0.100 ± 0.01^{a}
	MFC	3.125 ± 0.04^{c}	6.250 ± 0.08^{d}	3.120 ± 0.04^{c}	3.120 ± 0.04^{c}	12.500 ± 0.02^{d}	1.560 ± 0.20^{b}	1.500 ± 0.02^{b}	1.560 ± 0.00^{b}	0.300 ± 0.01^{a}	0.200 ± 0.01^{a}
P. funiculosum	MIC	1.560 ± 0.20^{b}	0.390 ± 0.01^{b}	1.560 ± 0.00^{b}	1.560 ± 0.02^{c}	0.390 ± 0.003^{a}	0.200 ± 0.01^{d}	0.400 ± 0.00^{a}	1.560 ± 0.00^{c}	2.500 ± 0.07^{c}	0.200 ± 0.01^{a}
	MFC	3.125 ± 0.04^{b}	0.780 ± 0.00^{b}	3.120 ± 0.04^{b}	3.120 ± 0.04^{c}	6.250 ± 0.08^{d}	0.780 ± 0.007^{b}	3.000 ± 0.07^{b}	3.125 ± 0.04^{c}	3.500 ± 0.10^{c}	0.250 ± 0.02^{a}
P. ochrochloron	MIC	0.780 ± 0.007^{c}	0.390 ± 0.003^{b}	1.560 ± 0.02^{d}	1.560 ± 0.02^{d}	1.560 ± 0.00^{d}	0.780 ± 0.007^{c}	0.400 ± 0.01^{b}	1.560 ± 0.02^{d}	0.200 ± 0.01^{a}	0.200 ± 0.003^{a}
	MFC	3.125 ± 0.04^{c}	0.780 ± 0.03^{c}	3.120 ± 0.04^{c}	3.120 ± 0.04^{e}	6.250 ± 0.08^{d}	1.560 ± 0.02^{d}	3.000 ± 0.07^{c}	3.125 ± 0.04^{e}	0.500 ± 0.01^{b}	0.250 ± 0.007^{a}
P. verucosum	MIC	6.250 ± 0.08^{d}	0.390 ± 0.07^{b}	3.120 ± 0.04^{c}	1.560 ± 0.02^{d}	6.250 ± 0.08^{d}	3.120 ± 0.04^{e}	3.000 ± 0.00^{c}	1.560 ± 0.02^{d}	1.000 ± 0.07^{b}	0.150 ± 0.007^{a}
	MFC	12.500 ± 0.20^{d}	0.780 ± 0.007^{b}	6.250 ± 0.08^{c}	6.250 ± 0.08^{d}	12.500 ± 0.20^{d}	6.250 ± 0.00^{a}	6.000 ± 0.10^{c}	6.250 ± 0.08^{a}	1.000 ± 0.007^{b}	0.200 ± 0.01^{a}

[a] Different letters in each row indicate significant differences between the extracts ($p < 0.05$). MIC, minimum inhibitory concentration; MBC, minimal bactericidal concentration; MFC, minimum fungicidal concentration.

Table 5 Effects of *Agaricus* spp. ethanolic extracts on biofilm formation of *P. aeruginosa* (PAO1) and disc-diffusion method for detection of anti-quorum (AQ) concentrations

	Biofilm formation* (%)			AQ (mm)		
Agents	0.5 MIC	0.25 MIC	0.125 MIC	0.125 MIC	0.25 MIC	0.5 MIC
Agaricus bisporus	31.37 ± 0.03^{b}	59.65 ± 0.06^{b}	101.65 ± 0.35^{b}	8.00 ± 1.0^{a}	9.67 ± 0.57^{a}	15.00 ± 2.0^{a}
Agaricus bitorquis	26.33 ± 0.46^{c}	64.70 ± 0.65^{a}	107.54 ± 1.70^{a}	8.30 ± 0.58^{a}	7.00 ± 1.0^{b}	8.70 ± 1.15^{b}
Agaricus campestris	47.45 ± 0.06^{a}	50.11 ± 0.03^{c}	98.78 ± 1.30^{c}	—	—	—
Agaricus macrosporus	13.06 ± 0.03^{d}	50.11 ± 0.30^{c}	89.37 ± 0.46^{d}	—	—	—
Ampicillin	69.16 ± 0.65^{a}	56.46 ± 0.46^{c}	92.16 ± 0.37^{c}	—	—	7.60 ± 0.6^{c}
Streptomycin	49.40 ± 0.46^{b}	70.97 ± 0.36^{a}	88.36 ± 0.42^{d}	—	15.0 ± 2.1^{a}	22.6 ± 2.3^{a}

Values are expressed as means ± SD. Different letters in each row indicate significant differences between the extracts ($p < 0.05$). *Biofilm formation values were calculated as: (mean A_{620} treated well)/(mean A_{620} control well) × 100. —No effect of AQ.

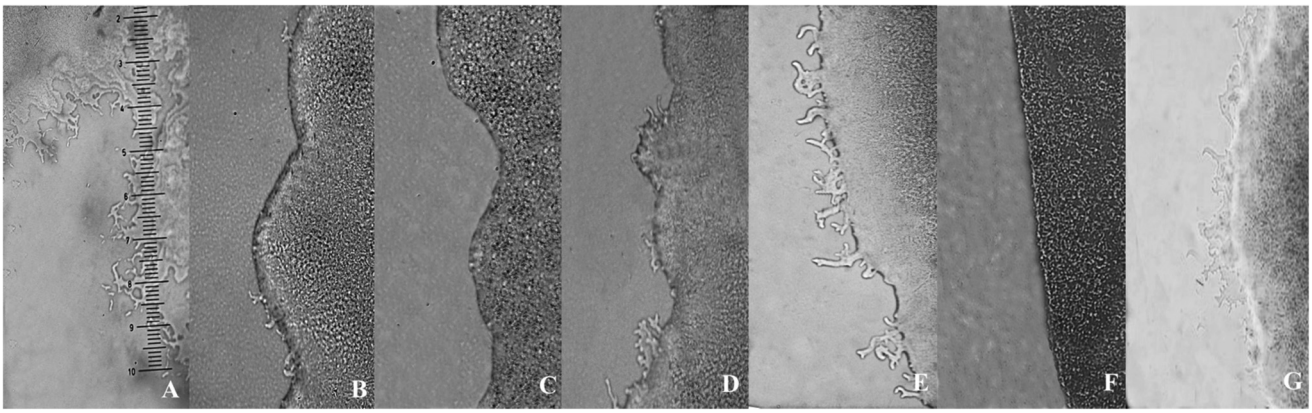

Fig. 1 Light microscopy of colony edges of *P. aeruginosa* in twitching motility plates, grown in the presence or absence of *Agaricus* spp. ethanolic extracts. A – *A. bisporus*, B – *A. bitorquis*, C – *A. campestris*, D – *A. macrosporus*, E – control P.a. 10^9, F – Streptomycin, G – Ampicillin.

Promising anti-quorum sensing compounds have been demonstrated to disrupt bacterial biofilms and make the bacteria more susceptible to antibiotics, and these compounds also provide the ability to reduce bacterial virulence factors as well as promote clearance of bacteria in infectious animal models. Many mechanisms of action have been proposed to interfere with the quorum sensing system such as inhibition of biosynthesis of autoinducer molecules, inactivation or degradation of the autoinducer, interference with the signal receptor, and inhibition of the genetic regulation system.[23]

In addition to QS, the initiation of biofilm formation by *P. aeruginosa* depends on two cell-associated structures: the flagellum and type IV pili.[21] The flagellum is responsible for swimming motility, while the type IV pili is responsible for twitching motility. Both types of motilities are important in the initial stages of biofilm formation by *P. aeruginosa*.[21] Therefore, we tried to determine whether our extract influences either one or both motilities. On swimming plates, the motile strain PAO1 was used as the 100% standard (control) for motility while the Petri dishes with the same strain plus *Agaricus* spp. extracts were compared with control. The extracts reduced the twitching motility of *P. aeruginosa*. The normal colonies of *P. aeruginosa*, *i.e.* in the absence of the extract, were flat with a rough appearance displaying irregular colony edges and a hazy zone surrounding the colony (Fig. 1E). The cells were in a very thin layer. After 2 days of incubation at ambient temperature, colony expansion occurred very rapidly due to twitching motility, and the control *P. aeruginosa* isolates produced swimming zones up to 100% and they were 14 mm in diameter. Bacteria that were grown with the *Agaricus* spp. extract solution were incapable of producing such a twitching zone and had almost round, smooth, regular colony edges, the flagella were reduced both in size and in numbers, and the colony diameter of the swimming zones was also reduced (18–32 mm) (Fig. 1A–D). All the *Agaricus* extracts reduced flagella with the exception of *A. bisporus* extract. The flagella reduction was achieved in the order *A. campestris* > *A. bitorquis* > *A. macrosporus* (Fig. 1C, 1B, 1D, respectively). Streptomycin completely reduced the flagella (Fig. 1F), while ampicillin did not affect the formation of flagella at all (Fig. 1G). The best twitching effect was achieved for *A. bitorquis* extract (18 mm) < *A. bisporus* (26 mm) < *A. campestris* (29 mm) < *A. macrosporus* (32 mm).

In summary, our study indicated that *Agaricus* extracts possessed antimicrobial, antibiofilm and anti-quorum sensing activity. Inhibition of bacterial quorum sensing offers a new strategy for the treatment of bacterial infections. The anti-

quorum sensing properties of these mushroom species may play an important role in antibacterial activity and offer an additional strategy for fighting bacterial infection.

In the present study a complete chemical characterization of the edible species *A. bisporus*, *A. bitorquis*, *A. campestris* and *A. macrosporus* was performed.

Data obtained for carbohydrates are in agreement with the values stated by different authors who reported mushrooms as good sources of carbohydrates and proteins as well as poor in fat and low caloric foods.[1,11] Mannitol and trehalose are very common sugars found in mushrooms as reported by several authors.[1,11,24] Due to the several applications of mannitol in food, pharmaceutical, medical, and chemical industries, the studied species are, also for this, considered valuable healthy foods; furthermore almost all species did not present other less healthy sugars like fructose or sucrose.[25] The phenolic profile of each one of the studied species was different. Nevertheless, the phenolic acid profile of the *A. bisporus* sample studied herein was similar to the one described for *A bisporus* samples from Finland[26] and Portugal.[15] Other *Agaricus* species presented different profiles: *p*-coumaric and cinnamic acids in *A. brasiliensis*[27] *p*-hydroxybenzoic and *p*-coumaric acids, and two related compounds, γ-L-glutaminyl-4-hydroxybenzene (GHB) and cinnamic acid, in *A. bohusii*.[28] All species seem to be excellent options with regard to their fatty acid content and composition; *A. macrosporus* may be a better choice since it has a lower percentage of SFA. Although organic acids are a product of the primary metabolism, some of these may also have bioactive properties such as malic acid that has been employed for the preparation of food additives and for the synthesis of various fine chemicals.[29,30] Different isoforms of tocopherols (α-, β-, γ- and δ-) were also found in the studied mushrooms, as also in other cultivated species.[31,32]

Among the studied mushrooms, *A. bisporus* is the best investigated especially regarding fatty acids and antioxidant activity.[33–36] The other species are not so well investigated; only a recent study on *A. bitorquis* chemical and nutritional composition is available[36]

A. campestris was the species with the highest antioxidant activity, probably due to its highest content in phenolic acids and tocopherols, known as powerful antioxidant molecules.[3]

Regarding the antibacterial and antifungal potential, the ethanolic extracts were more effective than the methanolic ones. Antimicrobial activity of three *Agaricus* species was also recently published by Ozturk *et al.*[37] who described effects of methanolic extracts against six species of Gram-positive bacteria, seven species of Gram-negative bacteria and two species of yeasts. The methanolic extract of *A. campestris* from India showed antimicrobial activity against seven bacterial species.[38]

4. Conclusion

Overall, the studied *Agaricus* species were found to be good sources of nutritional and bioactive compounds, and methanolic/ethanolic extracts showed antioxidant, antimicrobial and antiquorum properties. Thus, this study brings additional chemical and biochemical knowledge for these edible mushroom species, which can be applied in the food industry as natural preservatives.

Conflict of interest

The authors have no conflicts of interest.

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