Nutritional value, bioactive compounds, antimicrobial activity and bioaccessibility studies with wild edible mushrooms

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Running Tittle: Bioaccessibility and antimicrobial activity of phenolic acids and extracts of wild edible mushrooms

ABSTRACT

Wild mushrooms are important sources of nutrients and bioactive compounds, namely phenolic acids. After their ingestion, bioactive molecules have to be released from the matrix to be absorbed by the organism. In the present work, two wild edible mushrooms (*Volvopluteus gloiocephalus* and *Clitocybe subconnexa*) were studied for their nutritional value, detailed chemical composition and antimicrobial activity. Bioacessibility studies were also performed using the *in vitro* digestion of the crude powder, phenolic extracts and individual phenolic acids identified in the samples. The studied species proved to be rich sources of nutrients, minerals and bioactive molecules such as phenolic acids. The *in vitro* digestion conducted to a decrease in antibacterial activity, but not in antifungal and demelanizing properties. Nevertheless, in most of the cases, the analyzed samples presented higher antibacterial and antifungal activities than the standards. The bioactive molecules (phenolic acids) were found in higher concentrations in the phenolic extracts before *in vitro* digestion, which is agreement with the highest antibacterial activity revealed by these extracts. It should be highlighted that the phenolic acids were still bioavailable after digestion.

Keywords: Wild mushrooms; Chemical composition; Bioaccessibility; Antimicrobial activity

1. Introduction

Mushrooms are known worldwide as being valuable health foods, not only for their unique and subtle flavor, but also for their nutritional properties. The richness in carbohydrates, proteins, fibers, vitamins, and minerals, the presence of unsaturated fatty acids, and the poorness in fat as turned mushrooms in an excellent food choice to include in low caloric diets (Heleno, Barros, Sousa, Martins & Ferreira, 2010; Kalac, 2012; Reis, Barros, Martins & Ferreira, 2012).

Furthermore, these organisms are described as functional foods and/or a source of nutraceuticals due to biologically and physiologically active substances such as phenolic acids (Ferreira, Barros & Abreu, 2009). Mushroom extracts and their phenolic acids have been evaluated regarding antimicrobial activity, showing a very strong antibacterial, antifungal and also demelanizing properties; in several cases, even higher activity than the antibiotics/antifungals frequently used (Alves et al., 2012; Alves et al., 2013; Heleno et al., 2013). Thus, mushrooms could be introduced in our daily diet in order to help the organism in the prevention and combat against microbiological infections, taking advantage of the additive and synergistic effects of all their bioactive compounds (Alves et al., 2013).

Nevertheless, it is important to study the bioaccessibility of these extracts and compounds in order to guarantee the maintenance of the initial bioactivity, since the molecules can suffer structural modifications during the digestion and metabolism, before being absorbed by the intestine (Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso, 2013). Several easy and cheap methodologies such as *in vitro* digestions were developed and are described as being capable of mimetize the *in vivo* conditions in order to analyse the digestive stability of the food constituents (Bouayed, Hoffmann & Bohn, 2011).

In the present work, two wild and edible mushrooms (*Volvopluteus gloiocephalus* ((DC.) Vizzini, Contu & Justo) and *Clitocybe subconnexa* (Murril)) were studied for their nutritional value, detailed chemical composition and antimicrobial activity. To access their bioaccessibility, an *in vitro* digestion of the crude powder, phenolic extracts and individual phenolic acids identified in the two samples was carried out.

2. Materials and methods

2.1. Samples

Samples of *Volvopluteus gloiocephalus* ((DC.) Vizzini, Contu & Justo) and *Clitocybe subconnexa* (Murril), two wild edible mushrooms, were collected in Bragança (Northeast Portugal) during November of 2013. After authentications by Dr. Anabela Martins (Polytechnic Institute of Bragança), voucher specimens were deposited at herbarium of School of Agriculture of Polytechnic Institute of Bragança, Portugal. The specimens (for each species, three fruiting bodies in the same maturity stage) were immediately lyophilised (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh), mixed to obtain an homogenate sample and kept at -20 ºC 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 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 and standards of sugars (D-(+)-mannitol, D-(+)-trehalose), tocopherols (α-, β-, and γ-isoforms), organic acids (oxalic and fumaric acids), phenolic compounds (gallic, *p*-hydroxybenzoic, protocatechuic,

cinnamic and *p*-coumaric acids), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and dietary fiber enzyme kit (TDF-100A Kit), pepsin enzyme (P-7000), pancreatin enzyme (P-1750) and porcine bile (B-8631). Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). Micro (Fe, Cu, Mn and Zn) and macroelements (Ca, Mg, Na and K) standards (> 99% purity), as well $LaCl_2$ and CsCl (> 99% purity) were purchased from Merck (Darmstadt, Germany). Anthrone was obtained from Panreac Química (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Chemical composition

2.3.1. Proximate composition. The samples were analysed for proteins, fat, carbohydrates and ash, using the AOAC procedures (AOAC, 2012). The crude protein content (N × 4.38) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by the extraction of a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash and mineral content was determined by incineration at 550±15 ºC.

Total available carbohydrate (TAC) assay was performed by the Anthrone method as described by Osborne & Voogt (1986) using 0.25 g of sample. The samples were pre-treated with 13 mL of $HClO_4$ (52:100, v/v) and kept for 18 h in the dark. After this period, distilled water was added, the sample was filtered and the volume of the filtrate was adjusted to 100 mL. Finally, the solution was further diluted to 10%, and 5 mL of 0.1% anthrone solution (in H_2SO_4 73:100, v/v) was added. Samples were kept in a boiling water bath for 12 min where the anthrone reaction with sugars yielded a green colour, and absorbance was measured at 630 nm on a UV/Vis Spectrometer EZ210 (Perkin Elmer, Waltham, MA, USA) equipped with Lambda software PESSW ver. 1.2.

The absorbance of the sample solution was compared to a 10-100 µg/mL concentration range standard glucose calibration curve. TAC values were expressed as g/100 g of dry weight.

AOAC enzymatic-gravimetric methods (993.19 and 991.42) were used for soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) analysis (Latimer, 2012). In brief, freeze-dried samples were treated with alpha-amylase (heat-stable), protease and amyloglucosidase. The soluble and insoluble fractions were separated by vacuum filtration. Waste from the digests was dried at 100 °C. Total fiber is the sum of soluble and insoluble fiber fractions; both were expressed as g/100 g of dry weight.

Energy was calculated according to the following equation according to Regulation (EC) No. 1169/ 2011 of the European Parliament and of the Council, of 25 October 2011: Energy (kcal) = 4 × (g protein + g total available carbohydrate) + 2 × (g fiber) + 9 × (g fat).

2.3.2. Macro and microelements. Total mineral content (ashes) and mineral elements analysis were performed on dried samples. The method 930.05 of AOAC procedures was used; 500 mg of each sample were subject to dry-ash mineralization at 450°C±15 ºC. The residue of incineration was extracted with HCl (50% v/v) and HNO_3 (50% v/v) and made up to an appropriate volume with distilled water, where Fe, Cu, Mn and Zn were directly measured. An additional 1/10 (v/v) dilution of the sample extracts and standards was performed to avoid interferences between different elements in the atomic absorption spectroscopy: for Ca and Mg analysis in 1.16% La_2O_3/HCl (leading to $LaCl_2$); for Na and K analysis in 0.2% CsCl (Ruiz-Rodríguez et al., 2011; Fernández-Ruiz, Olives Barba, Sanchez-Mata, Camara & Torija, 2011). All measurements were performed in atomic absorption spectroscopy (AAS) with air/acetylene flame in Analyst

200 Perkin Elmer equipment (Perkin Elmer, Waltham, MA, USA), comparing absorbance responses with > 99.9% purity analytical standard solutions for AAS made with $Fe(NO_3)_3$, $Cu(NO_3)_2$, $Mn(NO_3)_2$, $Zn(NO_3)_2$, NaCl, KCl, $CaCO_3$ and Mg band. Triplicate mineralization and extractions were carried out on the same material. The results were expressed in mg per 100 g of dry weight.

2.3.3. Free sugars. Free sugars were determined by a High Performance Liquid Chromatography (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco, Easton, MD, USA), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Heleno, Barros, Sousa, Martins & Ferreira, 2009). Sugars were identified 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 commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.3.4. Fatty acids. Fatty acids were determined after a transesterification procedure as described previously by the authors (Heleno et al., 2009), 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). The results were expressed in relative percentage of each fatty acid.

2.3.5. Tocopherols. Tocopherols were determined following a procedure previously described by the authors (Heleno et al., 2010). Analysis was performed by HPLC (equipment described above), 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 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.

2.3.6. Organic acids. Organic acids were determined following a procedure previously described by the authors (Reis et al., 2013). Analysis was performed by ultra-fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). 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 g per 100 g of dry weight.

2.4. In vitro gastrointestinal model (dialysis) for bioaccessibility studies

Bioaccessibility was determined using 25 mL of aqueous solutions prepared from dry mushrooms powder (20 mg/mL), phenolic extracts (50 mg/mL) and phenolic acids (1 mg/mL). The phenolic extracts were previously prepared by extracting twice 1.5 g of

dry mushroom powder with methanol:water (80:20, *v/v*; 30 mL) at -20 ºC for 1.5 h; after a sonication step for 15 min, the extracts were filtered through Whatman No. 4 paper and dried (Barros, Dueñas, Ferreira, Baptista & Santos-Buelga, 2009).

The gastric digestion was simulated (Ramírez-Moreno, Marquês, Sánchez-Mata & Goñi, 2011; Heleno et al., 2015), adjusting the pH of each sample to 2, adding 150 mL of a pepsin solution (40 mg/mL of HCl 0.1M), and incubating the mixture in a water bath at 37 ºC for 2 h with stirring (60 osc/min). The intestinal digestion was then simulated, adding to the mentioned mixture a pancreatin/bile solution (5/25mg of pancreatin/bile per 1 mL of baking soda 0.1M). The mixture was then transferred to dialysis membranes, previously boiled in distilled water for 15 min. The dialysis membranes/mixture was then placed in to a flask containing 250 mL of baking soda pH 7.5 and incubated in a water bath at room temperature for 3 h with stirring (60 osc/min). After dialysis, the obtained final solution of baking soda pH 7.5 was frozen and lyophilized for evaluation of antimicrobial and demelanizing activity, and analysis of phenolic compounds.

2.5. Antimicrobial activity

Antibacterial activity was evaluated against Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973), following the procedure previously described by the authors (Soković, Glamočlija, Marin, Brkić & van Griensven, 2010). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined following the same reference. Streptomycin and

ampicillin were used as positive controls (1 mg/mL in sterile physiological saline). Antifungal activity was evaluated against *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061), and *Penicillium verrucosum* var. *cyclopium* (food isolate), following the procedure previously described by the authors (Soković & van Griensven, 2006). The minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations were determined following the same reference. 5% DMSO (solvent) was used as negative control. Bionazole and ketokonazole were used as positive controls (1-3000 µg/mL). The microorganisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research 'Siniša Stankovic', Belgrade, Serbia.

2.6. Demelanizing activity using micromycetes

All the microfungi tested for antifungal activity were used to evaluate the demelanizing activity of all the samples before and after *in vitro* digestion. The micromycetes were maintained on malt agar and the cultures were stored at 4°C. The fungal spores were submitted to the procedures previously described by the authors (Heleno et al., 2014). Determination of minimum demelanizing concentrations (MDC; the lowest concentration that provoked demelanization of fungal hyphae and conidia) was performed by a serial dilution technique. Samples from the control plate without added extracts were also stained and observed. Solution of 5% DMSO was used as a negative control.

2.7. Phenolic compounds analysis

The phenolic extracts were submitted to a liquid–liquid extraction with diethyl ether (2 x 20 mL) and ethyl acetate (2 x 20 mL). Anhydrous sodium sulphate was added to the combined organic phases and the extracts were filtrated through Whatman No. 4 paper, evaporated to dryness and re-dissolved in methanol:water (20:80, v/v). The extracts (1 ml) were filtered through a 0.22 μm disposable LC filter disk. Phenolic acids determination was performed using a Shimadzu 20A series ultra-fast liquid chromatograph (UFLC, Shimadzu, equipment described above) as previously described by Reis et al. (2013). Detection was carried out in a photodiode array detector (PDA), 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 100 g of dry weight.

2.8. Statistical analysis

Three samples were used for each preparation and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). In the case of proximate composition, free sugars, fatty acids, tocopherols, organic acids and minerals a student´s *t*-test was used because there were less than three groups. This treatment was carried out using SPSS v. 22.0 program.

3. Results and discussion

3.1. Chemical composition

The results concerning the proximate composition, free sugars and fatty acids of *V. gloiocephalus* and *C. subconnexa* are presented in **Table 1**. For both species, total dietary fiber (TDF) was the most abundant macronutrient, followed by proteins in *V.*

gloiocephalus, and total available carbohydrates (TAC) in *C. subconnexa*. Insoluble dietary fiber (IDF) predominated over soluble dietary fiber (SDF), which is in agreement with the existing reports in the literature (Kalac, 2012; Heleno et al., 2015). *C. subconnexa* revealed much higher contents in TAC contributing to the higher energetic value of this species, but presented lower amounts in proteins and fat.

Mannitol and trehalose were identified and quantified in both samples, but in higher concentration in *C. subconnexa*, especially mannitol, that contributed to the higher amount in total free sugars revealed by this species in which fructose was also found. Regarding the fatty acids, polyunsaturated fatty acids (PUFA) predominated over monounsaturated (MUFA) and saturated (SA) fatty acids due to the high contribution of linoleic acid (**Table 1**). The contents in palmitic and stearic acids were similar in both samples, whereas the contents in oleic acid were significantly higher in *C. subconnexa*, contributing to its higher amounts in MUFA. On the other hand, the contents in linoleic acids were higher in *V. gloiocephalus*, contributing to its higher amounts PUFA.

Concerning the tocopherols, α isoform was quantified in both samples, but in higher levels in *V. gloiocephalus*. Gamma- and beta-compounds were only found in this sample, contributing to its higher amounts of total tocopherols (**Table 2**). Oxalic and fumaric acids were identified in both samples, but in higher concentration in *C. subconnexa*.

Mineral elements (macro and microelements) were identified in both samples (**Table 3**). The macroelement K was the most abundant, followed by Na, Mg and finally Ca in both samples. Regarding the microelements, Fe was the most abundant, followed by Zn, Cu and Mn also in both samples. These results are in agreement with the reported in literature describing K as the major macroelement found in mushrooms (Mattila et al., 2001). *V. gloiocephalus* revealed much higher contents in K and Fe probably due to the

accumulation of these components caused by the adaptation to environmental condition (Kalac, 2012).

To the author's knowledge, this is the first report on the nutritional value and chemical characterization of *V. gloiocephalus*.

3.2. Bioaccessibility studies and antimicrobial activity of phenolic extracts (before and after in vitro digestion), and of in vitro digested mushrooms

The phenolic extracts of *V. gloiocephalus* and *C. subconnexa* showed higher antibacterial activity than the *in vitro* digested samples (either mushrooms or phenolic extracts; **Table 4**) that presented a similar behavior. Nevertheless, all the tested samples revealed higher antibacterial activity than ampicillin and, in almost of the cases, also higher than streptomycin. *Staphylococcus aureus* was the most susceptible bacteria to *V. gloiocephalus* phenolic extract, while *Salmonella typhimurium* was the most susceptible one to the *in vitro* digested samples (mushroom and phenolic extract). *Bacillus cereus* and *S. typhimurium* were the most sensitive bacteria to *C. subconnexa* phenolic extract; the *in vitro* digested mushroom presented the highest activity against *P. aeruginosa* and *S. typhimurium*; *P. aeruginosa* was also susceptible to the *in vitro* digested phenolic extract. *S. typhimurium* revealed to be the most sensitive bacteria to the tested samples, whereas *Micrococcus flavus* showed the highest resistance.

Regarding antifungal activity, the *in vitro* digested samples showed similar behavior (**Table 4**). *V. gloiocephalus* phenolic extract gave higher activity than digested samples, while the opposite was observed for *C. subconnexa*. All the samples exhibited higher antifungal activity than ketoconazole and, in almost of the cases, also than bifonazole. *Aspergillus ochraceus* and *Penicillium funiculosum* were the most susceptible fungi to *V. gloiocephalus* phenolic extract. *A. ochraceus* and *Trichoderma viride* were the most

sensitive to the *in vitro* digested mushroom. The *in vitro* digested phenolic extract was more efficient against *T. viride* and *P. funiculosum*. Regarding *C. subconnexa*, the phenolic extract was more effective against *T. viride* and *P. funiculosum*. *A. ochraceus* and *T. viride* were the most sensitive fungi to the *in vitro* digested mushroom. The *in vitro* digested phenolic extract was more effective against *T. viride*, *P. ochrochloron* and *P. verrucosum*. *T. viride* was the most susceptible fungi to the tested samples while the fungi belonging to the *Aspergillus* genus revealed the highest resistance (**Table 4**).

The phenolic acids identified in *V. gloiocephalus* and *C. subconnexa* extracts before and after *in vitro* digestion, and also in digested mushrooms are presented in **Table 5**. The phenolic extracts (before *in vitro* digestion) revealed the highest content in all the identified compounds, followed by the *in vitro* digested phenolic extracts. The *in vitro* digested mushrooms presented the lowest content, maybe due to the presence of phenolic compounds linked to other molecules (e.g., polysaccharides), that difficult the cross of the dialysis membrane (Bermúdez-Soto, Tomás-Barberán & García-Conesa, 2007; Rodríguez-Roque et al., 2013). However, it should be highlighted that the identified compounds are still bioavailable after digestion, although they are present in lower contents.

3.3. Bioaccessibility studies and antimicrobial activity of individual phenolic acids before and after in vitro digestion

Among the individual phenolic acids analyzed before *in vitro* digestion, cinnamic acid (CA) was the most active against all the bacteria, followed by *p*-hydroxybenzoic (HA) and *p*-coumaric (CoA) acids (**Table 6**). Gallic (GA) and protocatechuic (PA) acids showed the lowest antibacterial activity. Nevertheless, the phenolic acids revealed higher activity than ampicillin and streptomycin, against almost all the tested bacteria.

B. cereus was the most susceptible bacteria to the individual phenolic acids. After *in vitro* digestion, *p*-hydroxybenzoic (DHA) and protocatechuic (DPA) acids gave the highest antibacterial activity, followed by *p*-coumaric (DCoA) and cinnamic (DCA) acids; gallic acid (DGA) gave the lowest activity (**Table 6)**. *P. aeruginosa* was the most sensitive bacteria to the *in vitro* digested phenolic acids, being *M. flavus* the most resistant one. A decrease in the phenolic acids antibacterial activity was observed after *in vitro* digestion, which is in agreement with other reports describing structural modifications of the bioactive molecules due to the drastic pH variation (Bermúdez-Soto et al., 2007; Rodríguez-Roque et al., 2013). Still, the *in vitro* digested phenolic acids maintained a higher antibacterial activity than ampicillin and also than streptomycin against most of the bacteria (**Table 6**).

Among the individual phenolic acids, CA revealed the highest antifungal activity, followed by GA, PA and HA, with CoA presenting the lowest activity. *A. ochraceus* and *T. viride* were the most susceptible fungi to the individual phenolic acids, being *A. niger* and *A. fumigatus* the most resistant ones. The samples exhibited higher activity than the two standards used in the majority of the cases. For the *in vitro* digested phenolic acids, their antifungal activity was very similar. The most susceptible bacteria were *A. ochraceus* and *T. viride*, while *A. niger* was the most resistant one, as for the individual phenolic acids before digestion. The antifungal activity of the tested compounds was maintained after digestion, with the exception of DHA and DCA that presented lower activity. However, the *in vitro* digested phenolic acids also exhibited stronger antifungal activity than the standards, against the majority of the fungi (**Table 6**).

The demelanizing activity of the extracts and individual compounds was tested toward the same eight microfungi used for the antifungal assays. All the tested samples

revealed demelanizing properties but acting in different proportions against the different fungi. The phenolic extracts and individual phenolic acids showed the highest demelanizing abilities, presenting the *in vitro* digested samples a very similar behavior (Data not shown) to the one observed for the antifungal properties. Gallic acid was the most active compound; its effects were significant on *A. fumigatus, A. niger, P. funiculosum,* and *P. verrucosum* var. *cyclopium* (**Figure 1**). This compound provoked demelanizing effect on *A. fumigatus,* by lowering the amount of conidia and giving nude vesicle without conidia (**Figure 1A-C**). On *A. niger*, gallic acid reduced the number of spores and vesicles, and at higher concentrations it completely reduced spores and induced thinner hyphae (**Figure 1D-F**). Furthermore, this compound also reduced the number of spores on *P. funiculosum* and nude heads, and increasing the concentration, the hyphae became tiny and short **(Figure 1C-J)**. On *P. ochrochloron*, besides reducing the number of spores (**Figure 1K, L**), this compound provoked fialides without conidia (**Figure 1M, N**). Changes in both *Aspergillus* and *Penicillium* species are obvious and can be described as depigmentation, morphological changes of conidiphores-unusually small, number of heads and nude vesicles and fialides, when compared with control mycelium's (**Figure 1A-N**). The colored conidiophores of some *Aspergillus* and *Penicillium* species contain melanin that contribute to the virulence of pathogens of humans as well as those of food crops (Rosa, Vieira, Santiago & Rosa, 2010). Previous studies of demelanization activity of different mushroom extracts (*Ganoderma lucidum* and *Coprinopsis atramentaria*) showed strong effects on few microfungi, namely *A. niger* (Heleno et al. 2013; Heleno et al. 2014).

4. Conclusion

As far as we know this is the first report on the chemical characterization, antimicrobial activity and bioaccessibility of *V. gloiocephalus* and *C. subconnexa* mushrooms, extracts and phenolic acids. Although being low caloric foods (with low fat content and prevalence of PUFA), these two species proved to be rich sources of carbohydrates (e.g., fibers), proteins, minerals and also bioactive compounds (e.g., phenolic compounds and tocopherols). Regarding the antimicrobial activity, the phenolic extracts of *V. gloiocephalus* and *C. subconnexa* revealed higher activity than the *in vitro* digested samples (mushrooms or phenolic extracts); the observed antimicrobial activity decrease might be attributed to structural modifications caused by drastic pH variations and to the presence of phenolic acids linked to other molecules that difficult the cross through dialysis membrane. No significant differences were observed for the antifungal and demelanizing activities. CA was the most active phenolic acid against all the bacteria and also against fungi. It should be highlighted that, for almost all the cases, the analyzed samples presented higher antibacterial and antifungal activities than the standards. The concentration of the bioactive molecules (phenolic acids) decreased after *in vitro* digestion, which is agreement with the decrease observed for antibacterial activity. Nevertheless, the phenolic acids were still bioavailable, although in lower amounts.

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Table 1. Proximate composition, free sugars and fatty acids in *Volvopluteus gloiocephalus* ((DC.) Vizzini, Contu & Justo) and *Clitocybe subconnexa* (Murril).

Parameter	*Volvopluteus gloiocephalus*	*Clitocybe subconnexa*	Student´s *t*-test *p*-value
Ash (g/100 g dw)	14.19±0.07	5.98±0.04	<0.001
Total available carbohydrates	13.97 ± 0.34	27.35 ± 0.13	<0.001
Total Dietary Fiber	39.12 ± 0.29	38.74 ± 0.79	0.538
Insoluble fibre	37.08 ± 0.26	36.42 ± 1.19	0.003
Soluble fibre	2.04 ± 0.03	3.35 ± 0.36	0.394
Proteins (g/100 g dw)	19.66±0.14	7.42±0.25	<0.001
Fat (g/100 g dw)	4.62±0.04	1.02±0.09	<0.001
Energy (kcal/100 g dw)	366.34±0.05	381.18±0.23	<0.001
Fructose	nd	0.72±0.03	-
Mannitol	1.45±0.10	24.71±0.55	<0.001
Trehalose	1.92±0.11	6.00±0.01	<0.001
Total Sugars (g/100 g dw)	3.37±0.21	30.71±0.56	<0.001
C16:0	7.42±0.17	7.31±0.17	0.322
C18:0	2.09±0.07	2.15±0.08	0.205
C18:1n9c	25.96±0.02	42.50±1.19	<0.001
C18:2n6c	59.33±0.15	44.88±0.66	<0.001
SFA (percent)	12.56±0.17	11.00±0.46	0.001
MUFA (percent)	26.47±0.01	43.45±1.23	<0.001
PUFA (percent)	60.97±0.17	45.59±0.76	<0.001

dw- dry weight; Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c). SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids. The difference to 100% corresponds to other 23 less abundant fatty acids (data not shown).

Table 2. Tocopherols and organic acids in *Volvopluteus gloiocephalus* ((DC.) Vizzini, Contu & Justo) and *Clitocybe subconnexa* (Murril).

Compound	*Volvopluteus gloiocephalus*	*Clitocybe Subconnexa*	Students *t*-test *p*-value
α-Tocopherol	13.83±0.65	6.98±0.56	<0.001
β-Tocopherol	66.06±2.31	n.d.	-
γ-Tocopherol	20.20±2.74	n.d.	-
Total tocopherols (μg/100 g dw)	100.09±0.21	6.98±0.56	<0.001
Oxalic acid	0.37±0.03	2.64±0.07	<0.001
Fumaric acid	0.13±0.01	0.23±0.01	<0.001
Total organic acids (g/100 g dw)	0.50±0.03	2.87±0.07	<0.001

dw- dry weight. n.d.- not detected

Table 3. Macro and microelements in *Volvopluteus gloiocephalus* ((DC.) Vizzini, Contu & Justo) and *Clitocybe subconnexa* (Murril).

	Volvopluteus gloiocephalus	*Clitocybe Subconnexa*	Student´s *t*-test *p*-value
Macroelements (mg/100 g dw)			
Ca	73.52 ± 5.54	92.68 ± 0.96	<0.001
Mg	200.19 ± 14.57	148.86 ± 1.36	<0.001
Na	790.56 ± 67.26	722.04 ± 23.37	0.137
K	4692.55 ± 64.43	2023.25 ± 156.75	<0.001
Microelements (mg/100 g dw)			
Fe	69.91 ± 6.89	6.53 ± 0.18	<0.001
Cu	5.01 ± 0.44	5.22 ± 0.05	0.997
Mn	0.13 ± 0.01	0.10 ± 0.01	0.882
Zn	10.89 ± 0.12	6.37 ± 0.64	<0.001

dw- dry weight.

Table 4. Antimicrobial activity (MIC followed by MBC or MFC, μg/mL) of mushroom phenolic extracts before and after *in vitro* digestion, and of *in vitro* digested mushrooms.

	Volvopluteus gloiocephalus			***Clitocybe subconnexa***			**Standards**	
	Before *in vitro* digestion		After *in vitro* digestion	Before *in vitro* digestion		After *in vitro* digestion		
Bacteria	**Extract**	**Mushroom**	**Extract**	**Extract**	**Mushroom**	**Extract**	**Ampicillin**	**Streptomycin**
Staphylococcus	40	150	150	100	150	150	240	40
aureus	50	290	290	210	290	290	400	80
Bacillus	50	150	150	50	150	150	240	80
cereus	100	290	290	100	290	290	400	160
Micrococcus	100	170	150	150	210	150	240	160
flavus	210	330	290	210	420	290	400	320
Listeria	100	150	150	100	150	150	400	160
monocytogenes	210	290	290	210	290	290	480	320
Pseudomonas	50	100	100	70	90	90	800	160
aeruginosa	100	210	210	100	190	190	1280	320
Salmonella	50	90	90	50	90	90	400	160
typhimurium	210	190	190	100	190	210	480	320
Escherichia	100	150	150	100	170	150	240	160
coli	210	290	290	210	330	290	480	320
Enterobacter	100	100	150	150	100	150	400	400
cloacae	210	210	290	210	210	290	800	800
Fungi	**Extract**	**Mushroom**	**Extract**	**Extract**	**Mushroom**	**Extract**	**Bifonazole**	**Ketoconazole**
Aspergillus	63±10[b]	73	73	125	73	73	150	200
fumigatus	208±40	146	146	167	146	146	200	500
Aspergillus	42±10	52	63	83	57	63	150	150
ochraceus	83±20[c]	104	125	168	115	125	200	200
Aspergillus	42±10[b]	73	73	208	73	63	100	200
versicolor	125±10[b]	146	146	350	146	125	200	500
Aspergillus	167±40[a]	73	73	167	73	73	150	200
niger	317±40[a]	146	146	300	146	146	200	500
Trichoderma	26±10[b]	57	57	68	57	57	150	1000
viride	125±10[a]	115	115	94	115	115	200	1500
Penicillium	42±10[b]	63	57	73	63	63	200	200
funiculosum	83±20[c]	125	115	104	125	125	250	500
Penicillium	42±10[b]	63	63	83	63	57	200	1000
ochrochloron	125±10	125	125	125	125	115	250	1500
Penicillium	42±10[b]	63	63	83	63	57	200	1500
verrucosum	125±10	125	125	125	125	115	300	2000

MIC- minimal inhibitory concentrations; MBC- minimal bactericidal concentrations; MFC- minimal fungicidal concentrations.

Table 5. Phenolic acids (µg/100 g dw) in *Volvopluteus gloiocephalus* and *Clitocybe subconnexa* (phenolic extract and mushrooms) before and after *in vitro* digestion.

	Volvopluteus gloiocephalus			***Clitocybe subconnexa***		
	Before *in vitro* digestion	After *in vitro* digestion		Before *in vitro* digestion	After *in vitro* digestion	
Phenolic compounds	Phenolic extract	Mushroom	Phenolic extract	Phenolic extract	Mushroom	Phenolic extract
Gallic acid	74.15±3.46^{a}	20.08±0.89^{c}	60.88±2.84^{b}	55.62±1.43^{a}	12.33±0.15^{c}	24.62±0.58^{b}
p-Hydroxybenzoic acid	85.59±4.86^{a}	3.40±0.05^{c}	62.96±2.71^{b}	nd	nd	nd
p-Coumaric acid	171.21±0.06^{a}	20.83±0.10^{c}	75.94±0.36^{b}	155.03±1.22^{a}	nd	20.17±0.08^{b}
Protocatechuic acid	nd	nd	nd	184.39±4.04^{a}	31.93±0.24^{b}	33.25±0.49^{b}
Cinnamic acid	73.42±0.17^{a}	53.15±0.70^{c}	71.69±0.15^{b}	69.17±0.06^{a}	10.81±0.01^{b}	11.29±0.01^{b}
Total	404.37±1.28^{a}	97.46±1.75^{c}	271.47±5.76^{b}	464.21±6.74^{a}	55.08±0.08^{c}	89.33±1.17^{b}

dw- dry weight. In each row and for each mushroom species, different letters mean significant differences (p<0.05).

Table 6. Antimicrobial activity (MIC, followed by MBC or MFC, μg/mL) of individual phenolic acids found in *Volvopluteus gloiocephala* and *Clitocybe subconnexa*, before and after *in vitro* digestion.

	Before *in vitro* digestion					After *in vitro* digestion					Standards	
Bacteria	**HA***	**CA***	**CoA****	**GA**	**PA**	**DHA**	**DCA**	**DCoA**	**DGA**	**DPA**	**Ampicillin**	**Streptomycin**
Staphylococcus	3	2	90	90	100	150	150	170	190	150	240	40
aureus	7	3	180	190	210	290	290	333	380	290	400	80
Bacillus	3	2	50	50	60	150	150	170	190	100	240	80
cereus	7	3	90	100	130	290	290	333	380	210	400	160
Micrococcus	15	12	140	111	140	150	170	170	190	150	240	160
flavus	30	30	180	230	210	290	330	333	380	290	400	320
Listeria	30	7	50	150	100	150	150	150	190	150	400	160
monocytogenes	60	60	180	290	210	290	290	290	380	290	480	320
Pseudomonas	3	0.7	50	70	60	100	100	80	100	100	800	160
aeruginosa	7	2	180	150	130	210	210	170	210	210	1280	320
Salmonella	3	2	90	50	60	100	100	100	190	100	400	160
typhimurium	7	3	180	100	130	210	210	210	380	210	480	320
Escherichia	3	7	90	90	140	150	170	130	190	100	240	160
coli	60	60	180	190	210	290	333	250	380	210	480	320
Enterobacter	6	2	90	190	100	150	100	100	190	100	400	400
cloacae	7	3	180	250	210	290	210	210	380	210	800	800
Fungi	**HA***	**CA***	**CoA****	**GA**	**PA**	**DHA**	**DCA**	**DCoA**	**DGA**	**DPA**	**Bifonazole**	**Ketoconazole**
Aspergillus	120	7	130	70	70	60	60	60	60	60	150	200
fumigatus	250	20	250	150	150	130	130	130	130	130	200	500
Aspergillus	20	7	130	60	60	50	50	50	50	50	150	150
ochraceus	70	30	250	111	111	111	111	100	111	111	200	200
Aspergillus	3	7	60	60	60	60	60	60	70	70	100	200
versicolor	30	60	130	130	130	130	130	130	150	150	200	500
Aspergillus	30	30	250	70	70	70	80	70	70	80	150	200
niger	70	60	450	150	150	150	140	150	150	150	200	500
Trichoderma	7	20	130	60	60	50	50	50	50	50	150	1000
viride	20	30	250	111	111	111	111	111	111	111	200	1500
Penicillium	30	20	250	60	60	60	60	60	60	60	200	200
funiculosum	70	60	450	130	111	130	111	130	111	111	250	500
Penicillium	60	30	130	60	60	60	60	60	60	60	200	1000
ochrochloron	70	60	250	130	130	130	111	130	130	130	250	1500
Penicillium	60	7	130	60	60	60	60	60	60	60	200	1500
verrucosum	070	30	250	130	130	130	130	111	111	130	300	2000

MIC- minimal inhibitory concentrations; MBC- bactericidal concentrations; MFC- fungicidal concentrations; HA- *p*-Hydroxybenzoic acid; CA- Cinnamic acid; CoA- *p*-Coumaric acid; PA- Protocatechuic acid; DHA- *In vitro* digested *p*-hydroxybenzoic acid; DCA- *In vitro* digested cinnamic acid; DCoA- *In vitro* digested *p*-coumaric acid; GA- Gallic acid; DGA- *In vitro* digested gallic acid; DPA- *In vitro* digested protocatechuic acid. * Previously published in Heleno et al. 2013. ** Previously published in Heleno et al., 2014.

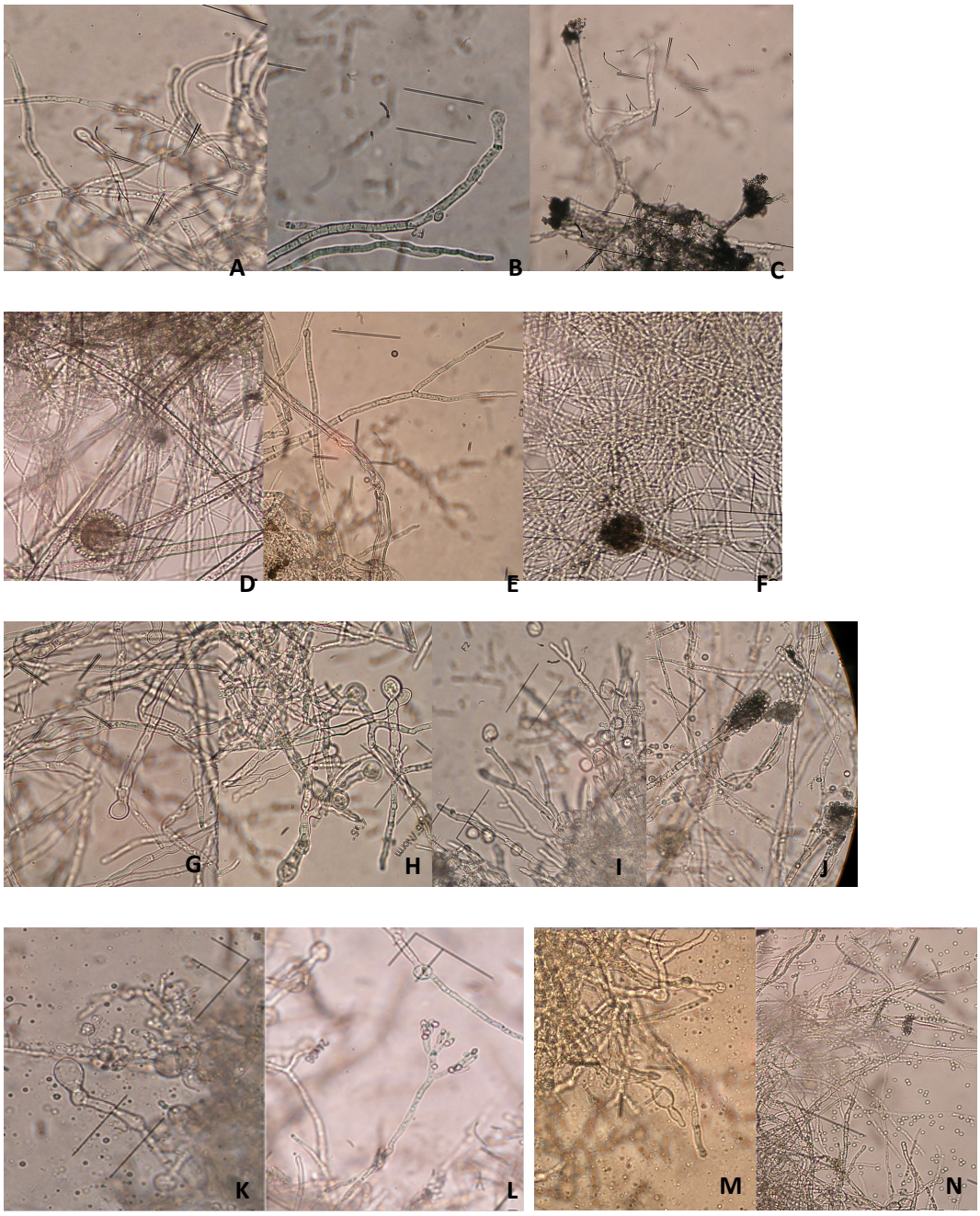

Figure 1. Demelanizing activity of gallic acid against different fungi at different concentrations: **A**- 31.25 µg/mL on *A. fumigatus*, **B**- 62.5 µg/mL on *A. fumigatus*, **C**- control *A. fumigatus*, **D**- 62.5 µg/mL on *A. niger*, **E**- 125 µg/mL on *A. niger*, **F**- control *A. niger*, **G**- 15.6 µg/mL on *P. funiculosum*, **H**- 31.25 µg/mL on *P. funiculosum*, **I**- 62.5 µg/mL on *P. funiculosum*, **J**- control *P. funiculosum*, **K**- 31.25 µg/mL on *P.*

ochrochloron, **L**- control *P. ochrochloron,* **M**- 31.25 µg/mL on *P. veruccosum* var. *cyclopium*, **N**- control *P. veruccosum* var. *cyclopium*.