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Effect of Fruiting Body Maturity Stage on Chemical Composition and Antimicrobial Activity of *Lactarius* sp. Mushrooms

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The effects of fruiting body maturity on chemical composition and antimicrobial activity of the wild mushrooms, *Lactarius deliciosus* and *Lactarius piperatus*, were evaluated. Immature and mature samples were analyzed for proximate constituents (moisture, fat, crude protein, ash, carbohydrates), nutritional value, and individual composition in fatty acids and sugars. Protein content, MUFA (monounsaturated fatty acids), and PUFA (polyunsaturated fatty acids) percentages increased with the fruiting body maturity stage, while carbohydrate and SFA (saturated fatty acids) content decreased. The maturity stage did not significantly affect the individual sugar profile. The antimicrobial activity of the mushrooms was screened against Gram positive and Gram negative bacteria and fungi, and correlated to the amounts of phenols, flavonoids, ascorbic acid, β-carotene, and lycopene present in the immature and mature fruiting bodies. Mature fruiting bodies with mature spores presented lower antimicrobial activity, which was in agreement with the bioactive compound content found in those samples.

KEYWORDS: Antimicrobial activity; chemical composition; fruiting body maturity; *Lactarius deliciosus*; *Lactarius piperatus*

INTRODUCTION

In recent years, multiple drug resistance in human pathogenic microorganisms has developed, due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various plants which are good sources of novel antimicrobial chemotherapeutic agents (*1, 2*). Mushrooms are rich sources of natural antibiotics, and therefore mushrooms extracts have been investigated for their antimicrobial activity. The capacity of the Chinese shiitake mushroom (*Lentinus edodes*) to increase the host resistance to bacterial and viral infections (*3*) was demonstrated, and several compounds extracted from this mushroom revealed antifungal and antibacterial activity (*4–8*). *Laetiporus sulphureus* antimicrobial activity was also reported and correlated to the phenol and flavonoid contents (*2*), similarly to the study performed by us on the antimicrobial properties of phenolic extracts of other Portuguese wild edible mushroom species (*Lactarius deliciosus*, *Sarcodon imbricatu*s, and *Tricholoma portentosum*) (*9*). Additionally, many of the externalized secondary metabolites (extracellular secretions by the mycelium) combat bacteria (*10, 11*), viruses (*12, 13*), and protozoa (*14, 15*). Compounds such as phenolics (phenolic acids and flavonoids), carotenoids, tocopherol, and ascorbic acid are important protective agents for human health (*16*).

Besides their pharmacological (*17, 18*) characteristics, wild mushrooms are becoming more and more important in our diet for their nutritional value, including high protein and low fat/energy contents (*19–24*). Dikeman et al. (*25*) reported the effects of the stage of maturity and cooking on the chemical composition of mushroom species commonly cultivated and consumed in the United States. Nevertheless, there are no detailed studies on the influence on fatty acid and sugar profiles, particularly on mushrooms from Northeast Portugal, one of the European regions with higher wild edible mushroom diversity. Also, none of the existing reports on antimicrobial activity and bioactive compounds of mushrooms indicated the stage of development of the fruiting bodies selected for the studies. It is known that the quality of a nutraceutical is dependent on the chemical composition of the fruiting body (*26*), and therefore, it is important to develop studies to evaluate bioactive properties and chemical composition at different stages of fruiting body maturity either for potential use on the preparation of nutraceutical or to achieve the ideal stage for consumption concerning nutritional value.

In this study, we examined the evolution of chemical composition and antimicrobial activity of Portuguese *Lactarius* species (*L. deliciosus* and *L. piperatus*), in three stages of fruiting body maturity. Chemical composition evaluation included

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Figure 1. *Lactarius piperatus* (**A**) and *Lactarius deliciosus* (**B**) fruiting bodies in different stages of maturity: (SI) immature (cap diameter less than 4.5 cm), (SII) mature (cap diameter between 4.5 and 7 cm) with immature spores, and (SIII) mature (cap diameter higher than 7 cm) with mature spores.

moisture, fat, protein, ash, carbohydrates, and nutritional value determination, individual fatty acid and sugar profiles, as well as bioactive compound determination such as phenols, flavonoids, and carotenoids. Antimicrobial activity was screened against Gram positive and Gram negative bacteria and fungi, and correlated to the bioactive compounds present in the extracts. The entire extracts were used to achieve health benefits due to the additive and synergistic effects of all the bioactive compounds present in the extracts, in order to stimulate the use of mushrooms not only for their nutritional properties but also as a source for the development of drugs and nutraceuticals.

MATERIALS AND METHODS

Samples/Fruiting Bodies Selection. Wild fruiting bodies of *Lactarius deliciosus* (L.) Gray and *Lactarius piperatus* (L.) Pers. were obtained, respectively, under live pine (*Pinus* sp.) in Autumn 2005 and oak (*Quercus pyrenaica*) trees in Spring 2006, in Bragança (Northeast of Portugal). Taxonomic identification was made according to several authors (*27, 28*) and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. The fruiting bodies were harvested and dried in different stages of maturity: immature and mature (with different cap diameters). The mature stage was further characterized into immature spores and mature spores according to **Figure 1**.

Standards and Reagents. All reagents were of analytical grade purity: methanol and diethyl ether were supplied by Laboratory-Scan (Lisbon, Portugal); toluene from Riedel-de-Haen; sulfuric acid from Fluka. The fatty acid methyl ester (FAME) reference standard mixture 37 (fatty acids C4 to C24; standard 47885-U) was from Supelco (Bellefonte, PA) and purchased from Sigma (St. Louis, MO), as well as other individual fatty acid isomers, gallic acid, and (+)-catechin. The standards used in the antimicrobial activity assays, ampicillin and cycloheximide, were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

Chemical Composition. Samples of mushrooms were analyzed for chemical composition (moisture, protein, fat, carbohydrates, and ash) using the AOAC procedures (*29*). The crude protein content (N × 4.38) of the samples was estimated by the macroKjeldahl method according to Léon-Guzmán et al. (*30*); the crude fat was determined by extracting a known weight of powdered mushroom sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C; reducing sugars were determined by the DNS (dinitrosalicylic acid) method. Total carbohydrates were calculated by difference: Total carbohydrates = 100 − (g moisture + g protein + g fat + g ash). Total energy was calculated according to the following equations: Energy (kcal) = 4 × (g protein +g carbohydrate) + 9 × (g lipid).

Fatty Acid Composition. Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GLC-FID)/capillary column based on the transesterification procedure described by us in previously reports (*31*). Fatty acids were methylated with 5 mL of methanol/sulfuric acid/toluene 2:1:1 (v:v), during at least 12 h in a bath at 50 °C and 160 rpm; then, 5 mL of deionized water was added, to obtain phase separation; the FAME were recovered with 5 mL of diethyl ether by shaking in vortex, and the upper phase was passed through a microcolumn of sodium sulfate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 μm nylon filter from Milipore. The fatty acid profile was analyzed with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID), and a Macherey–Nagel column (30 m × 0.32 mm i.d. × 0.25 μm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 10 °C/min ramp to 240 °C and held for 11 min. The carrier gas (hydrogen) flow rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. For each analysis, 1 μL of the sample was injected in GC. 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) and expressed in relative percentage of each fatty acid.

Sugar Composition. Free sugars were determined by high-performance liquid chromatography coupled to a refraction index detector (HPLC-RI) based on the method used by us in previous reports (*24, 31*). Dried powder (1.0 g) was extracted with 40 mL of 80% aqueous ethanol at 80 °C for 30 min. The resulting suspension was centrifuged at 15,000 *g* for 10 min. The supernatant was concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL. Soluble sugars were determined by using HPLC (Knauer, Smartline system) at 35 °C. The HPLC system was equipped with a Knauer Smartline 2300 RI detector and with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer). The mobile phase was acetonitrile/deionized water, 7:3 (v/v) at a flow rate of 1.25 mL/min. The results are expressed in g/100 g of fresh weight, calculated by internal normalization of the chromatographic peak area. Sugar identification was made by comparing the relative retention times of sample peaks with standards. The sugar standards used for identification were purchased from Sigma Chemical Co. (St. Louis, MO): L-(+)-arabinose, D-(−)-fructose, D-(+)-galactose, D-(+)-glucose anhydrous, lactose

Table 1. Moisture, Proximate Chemical Composition (g/100 g of Fresh Weight), and Energetic Value (kcal/100 g of Fresh Weight) of the Mushrooms at Different Stages of Maturity (Mean ± SD; $n = 3$)[a]

samples	moisture	total fat	crude protein	ash	carbohydrates	reducing sugars	energy
L. deliciosus SI	90.91 ± 0.27 a	0.47 ± 0.21 a	1.29 ± 0.02 c	1.30 ± 0.03 b	7.32 ± 0.99 a	0.05 ± 0.00 b	38.68 ± 2.60 a
L. deliciosus SII	90.55 ± 0.58 ba	0.37 ± 0.10 a	2.07 ± 0.08 b	1.44 ± 0.02 a	5.21 ± 0.18 b	0.18 ± 0.02 a	32.41 ± 1.36 b
L. deliciosus SIII	89.61 ± 0.31 b	0.55 ± 0.10 a	4.41 ± 0.07 a	1.54 ± 0.07 a	2.96 ± 0.50 c	0.04 ± 0.00 b	34.39 ± 2.29 ba
L. piperatus SI	88.04 ± 1.05 a	0.97 ± 0.16 a	0.82 ± 0.02 c	0.83 ± 0.05 a	9.35 ± 1.10 a	0.05 ± 0.00 b	49.41 ± 3.82 a
L. piperatus SII	89.28 ± 0.33 a	0.69 ± 0.32 a	1.58 ± 0.03 b	0.94 ± 0.15 a	7.51 ± 0.18 a	0.22 ± 0.02 a	42.53 ± 3.50 ba
L. piperatus SIII	89.90 ± 1.17 a	0.70 ± 0.20 a	4.12 ± 0.14 a	0.99 ± 0.20 a	4.29 ± 1.36 b	0.06 ± 0.00 b	39.96 ± 4.29 b

[a] In each row and for each species, different letters mean significant differences ($p < 0.05$).

1-hydrate, maltose 1-hydrate, D-(+)-mannitol, D-(+)-mannose, D-(+)-melezitose, D-(+)-melibiose monohydrate, L-(+)-rhamnose monohydrate, D-(+)-sucrose, D-(+)-trehalose, and D-(+)-xylose.

Antimicrobial Activity. *Sample Preparation.* The samples (~3 g) were extracted by stirring with 100 mL of methanol at 25 °C at 150 rpm for 24 h and filtered through Whatman no 4 paper. The residue was then extracted with two additional 100 mL portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 °C to dryness and redissolved in DMSO at a concentration of 50 mg/mL, and stored at 4 °C for further use.

Microorganisms and Culture Conditions. Microorganisms labeled CECT were obtained from the Spanish-type culture collection (CECT) of Valencia University, while microorganisms labeled ESA were clinically isolated strains from different biological fluids, identified by Microbiology Laboratory of Escola Superior Agrária de Bragança. Gram+ (*Bacillus cereus* CECT 148, *B. subtilis* CECT 498, *Staphylococus aureus* ESA 7 isolated from pus) and Gram– (*Escherichia coli* CECT 101, *Pseudomonas aeruginosa* CECT 108, *Klebsiella pneumoniae* ESA 8 isolated from urine) bacteria, and fungi (*Candida albicans* CECT 1394, *Cryptococcus neoformans* ESA 3 isolated from vaginal fluid) were used to screen antimicrobial activity of samples. Microorganisms were cultured aerobically at 37 °C (Scientific 222 oven model, 2003) in nutrient agar medium for bacteria, and at 30 °C (Scientific 222 oven model, 2003) in sabouraud dextrose agar medium for fungi.

Test Assays for Antimicrobial Activity. A screening of antibacterial activities against the Gram− and Gram+ bacteria and fungi was performed, and the minimal inhibitory concentration (MIC) was determined by an adaptation of the agar streak dilution method based on radial diffusion (*9*). Suspensions of the microorganisms were prepared to contain approximately 10^8 cfu/mL, and the plates containing agar medium were inoculated (100 μL). A 50 μL volume of each sample was placed in a hole (depth 3 mm, diameter 4 mm) made in the center of the agar. Under the same conditions, different DMSO solutions of ampicillin (antibacterial) and cycloheximide (antifungal) were used as standards. DMSO was chosen as the best solvent after comparative toxicity assays which proved its nontoxicity. The MIC was considered to be the lowest concentration of the tested sample able to inhibit the growth of bacteria or fungi, after 24 h. The inhibition halos corresponding to the MICs were measured using a ruler, with an accuracy of 0.5 mm. Each inhibition halo was measured three times (three different plates) and the average was considered. A control using only inoculation was also carried out.

Determination of Bioactive Components. Phenolic compounds in the mushroom extracts were estimated by a colorimetric assay, based on procedures described by Singleton and Rossi with some modifications (*32*). Briefly, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and the volume adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve (0.01–0.4 mM; $Y = 2.8557X – 0.0021$; $R^2 = 0.9999$) and the results were expressed as milligrams of gallic acid equivalents (GAEs) per gram of extract.

Flavonoid contents in the extracts were determined by a colorimetric method described by Jia et al. (*33*) with some modifications. The mushroom extract (250 μL) was mixed with 1.25 mL of distilled water and 75 μL of a 5% $NaNO_2$ solution. After 5 min, 150 μL of a 10% $AlCl_3 \cdot H_2O$ solution was added. After 6 min, 500 μL of 1 M NaOH and 275 μL of distilled water were added to the mixture. The solution was mixed well and the intensity of the pink color was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.022–0.34 mM; $Y = 0.9629X – 0.0002$; $R^2 = 0.9999$) and the results were expressed as milligrams of (+)-chatequin equivalents (CEs) per gram of extract.

Ascorbic acid was determined according to the method of Klein and Perry (*34*). The dried methanolic extract (100 mg) was extracted with 10 mL of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman no 4 filter paper. The filtrate (1 mL) was mixed with 9 mL of 2,6-dichlorophenolindophenol and the absorbance was measured within 30 min at 515 nm against a blank. The content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.020–0.12 mg/mL; $Y = 3.4127X - 0.0072$; $R^2 = 0.9905$) and the results were expressed as milligrams of ascorbic acid per gram of extract.

β-Carotene and lycopene were determined according to the method of Nagata and Yamashita (*35*). The dried methanolic extract (100 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman no 4 filter paper. The absorbance of the filtrate was measured at 453, 505, and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 mL) = −0.0458 A663 + 0.372 A505 − 0.0806 A453; β-carotene (mg/100 mL) = 0.216 A663 − 0.304 A505 + 0.452 A453. The results were expressed as micrograms of carotenoid per gram of extract.

Statistical Analysis. For each one of the fruiting body maturity stages, three samples were analyzed, and in addition, all the assays were carried out in triplicate. The results are expressed as mean values and standard error (SE) or standard deviation (SD). The effects of the fruiting body maturity stage on the chemical composition and antimicrobial activity were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. This treatment was carried out using the *SAS v 9.1.3* program.

RESULTS AND DISCUSSION

Effects on the Chemical Composition. The results of the chemical composition and estimated energetic value obtained for the wild mushroom species are shown in **Table 1**. Concerning moisture, fat, and ash contents, no significant differences were obtained among the samples at different stages of fruiting body maturity. The most abundant macronutrients (carbohydrates and proteins) presented opposite evolutions along the maturity of fruiting body. Carbohydrates significantly decreased in both species (7.32 to 2.96 g/100 g for *L. deliciosus* and 9.35 to 4.29 g/100 g for *L. piperatus*), while proteins increased (1.29 to 4.41 g/100 g for *L. deliciosus* and 0.82 to 4.12 g/100 g for *L. piperatus*). The decrease in carbohydrate content may be explained by their energetic role, being catabolized for energy production along the mushroom growth. Otherwise, the increase in protein (mainly structural compounds) content may be due to the protein synthesis inherent to mushrooms maturity (*36*).

On the basis of the proximate analysis, it can be calculated that 100 g of fresh *L. piperatus* ensures higher energy values than *L. deliciosus*, in the same stage of maturity. The highest values are guaranteed by immature fruiting bodies (**Table 1**),

Table 2. Fatty Acid Composition (Percent) of the Mushrooms at Different Stages of Maturity (Mean ± SD; $n = 3$)[a]

	L. deliciosus SI	*L. deliciosus* SII	*L. deliciosus* SIII	*L. piperatus* SI	*L. piperatus* SII	*L. piperatus* SIII
C10:0	4.67 ± 0.76	5.46 ± 0.58	3.84 ± 0.81	0.03 ± 0.00	0.06 ± 0.02	0.09 ± 0.02
C11:0	*nd*	*nd*	*nd*	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.01
C12:0	0.01 ± 0.00	0.41 ± 0.01	0.50 ± 0.09	0.01 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C14:0	0.32 ± 0.07	0.18 ± 0.03	0.23 ± 0.03	0.11 ± 0.01	0.17 ± 0.02	0.24 ± 0.03
C14:1	0.17 ± 0.03	0.11 ± 0.01	0.05 ± 0.01	*nd*	*nd*	*nd*
C15:0	0.32 ± 0.06	0.32 ± 0.02	0.32 ± 0.02	0.28 ± 0.03	0.37 ± 0.03	0.51 ± 0.04
C16:0	4.19 ± 0.36	5.40 ± 0.13	7.02 ± 0.34	1.10 ± 0.13	2.16 ± 0.10	5.15 ± 0.64
C16:1	0.11 ± 0.01	0.18 ± 0.01	0.30 ± 0.02	0.03 ± 0.00	0.06 ± 0.00	0.12 ± 0.02
C17:0	0.17 ± 0.01	0.15 ± 0.00	0.11 ± 0.00	0.16 ± 0.01	0.14 ± 0.00	0.12 ± 0.01
C17:1c	*nd*	*nd*	*nd*	0.01 ± 0.00	0.08 ± 0.02	0.18 ± 0.07
C18:0	61.71 ± 2.43	51.29 ± 0.30	37.82 ± 1.08	81.46 ± 1.45	74.97 ± 0.51	54.53 ± 2.84
C18:1n9c	5.50 ± 0.67	12.51 ± 0.40	22.93 ± 0.44	6.58 ± 0.72	11.35 ± 0.40	26.99 ± 2.33
C18:2n6c	21.43 ± 0.84	22.70 ± 0.34	25.37 ± 0.10	6.66 ± 0.59	6.86 ± 0.04	8.57 ± 0.17
C18:3n6	0.26 ± 0.10	0.18 ± 0.03	0.15 ± 0.04	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
C18:3n3	0.34 ± 0.04	0.37 ± 0.01	0.48 ± 0.02	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C20:0	0.17 ± 0.01	0,18 ± 0.01	0.15 ± 0.01	2.40 ± 0.03	2.18 ± 0.03	1.56 ± 0.14
C20:1c	0.09 ± 0.00	0,03 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00
C20:2c	0.04 ± 0.01	0,04 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C20:3n3 + C21:0	0.02 ± 0.00	0,02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
C22:0	0.15 ± 0.06	0,16 ± 0.04	0.21 ± 0.02	0.39 ± 0.01	0.53 ± 0.03	0,52 ± 0.02
C23:0	0.07 ± 0.02	0,05 ± 0.01	0.06 ± 0.01	0.17 ± 0.02	0.17 ± 0.01	0.48 ± 0.10
C24:0	0.26 ± 0.06	0,27 ± 0.10	0.35 ± 0.02	0.53 ± 0.02	0.76 ± 0.02	0.76 ± 0.02
total SFA	72.04 ± 3.83 a	63.87 ± 1.22 b	50.60 ± 2.42 c	86.62 ± 1.72 a	81.53 ± 0.77 b	64.00 ± 3.86 c
total MUFA	5.87 ± 0.72 c	12.83 ± 0.42 b	23.32 ± 0.47 a	6.63 ± 0.73 c	11.51 ± 0.43 b	27.33 ± 2.42 a
total PUFA	22.09 ± 0.99 c	23.30 ± 0.38 b	26.06 ± 0.17 a	6.74 ± 0.59 b	6.94 ± 0.05 b	8.64 ± 0.18 a

[a] In each row and for each species, different letters mean significant differences ($p < 0.05$).

Table 3. Sugar Composition (g/100 g of Fresh Weight) of the Mushrooms in Different Stages of Maturity (Mean ± SD; $n = 3$)[a]

	mannitol	trehalose	total sugars
L. deliciosus SI	1.24 ± 0.01 b	0.35 ± 0.00 a	1.59 ± 0.01 a
L. deliciosus SII	1.34 ± 0.10 ba	0.38 ± 0.03 a	1.72 ± 0.13 a
L. deliciosus SIII	1.50 ± 0.07 a	0.24 ± 0.01 b	1.74 ± 0.08 a
L. piperatus SI	0.36 ± 0.07 a	1.16 ± 0.10 a	1.51 ± 0.12 a
L. piperatus SII	0.41 ± 0.02 a	0.83 ± 0.20 a	1.24 ± 0.18 a
L. piperatus SIII	0.43 ± 0.04 a	1.20 ± 0.17 a	1.63 ± 0.21 a

[a] In each row and for each species, different letters mean significant differences ($p < 0.05$).

despite the similarity in the results obtained for the other maturity stages; concerning dry matter, there are no significant differences between immature and mature fruiting bodies. This is in agreement with other authors (*25*) who reported that the stage of maturity did not affect proximate constituents of the mushrooms in a consistent manner.

The results for fatty acid composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) of the studied mushrooms are shown in **Table 2**. For both species, the major fatty acid found was stearic acid (C18:0), followed by linoleic acid (C18:2) and oleic acid (C18:1). Besides those three main fatty acids, nineteen more were identified and quantified. Saturated fatty acids (SFA) predominate over the unsaturated fatty acids (UFA) in all the stages of fruiting body maturity. SFA content decreased with the increase of fruiting body maturity due to the decrease in stearic acid percentage. Otherwise, the increase in linoleic and oleic acids contribute to the overall increase in PUFA and MUFA contents, respectively.

With regard to sugar composition (**Table 3**), mushrooms with fruiting bodies in different maturity stages showed some homogeneity. All of them presented mannitol and trehalose as main sugars. Mannitol was the most abundant sugar for *L. deliciosus* mushrooms with trehalose predominant in *L. piperatus*samples. A slight increase in mannitol content was observed for mature stages. Sugar alcohols, particularly mannitol, function to provide support and expansion of the fruit body, possibly explaining the increase in sugar alcohol concentration with maturity of the fruiting body as observed in the current study.

No significant differences between total sugars of immature or mature stages were observed, with only a slight increase observed in mature fruiting bodies. In fact, other authors (*25*) observed for different species (*Grifola frondosa* and *Lentinus edodes*) a total free monosaccharide increase in mature mushrooms, as compared with immature mushrooms. Nevertheless, the same results were not observed for the *Agaricus bisporus* mushroom.

Effects on the Antimicrobial Activity. Table 4 shows phenol, flavonoid, ascorbic acid, and carotenoid concentrations obtained in *Lactarius* species in different stages of fruiting body maturity. Whereas total phenols were the major bioactive components found in the extracts, ascorbic acid was found in small amounts (0.08–0.16 mg/g), and β-carotene and lycopene were only found in vestigial amounts (<49 μg/g). The mature stage where the fruiting bodies presented mature spores (stage III) revealed a lower content of bioactive compounds. For both species, the amounts found in stage III significantly ($p < 0.05$) decreased when compared with the contents found in the first stages. Probably, those compounds are involved in defense mechanisms inherent to the aging process (presence of mature spores), resulting in the lowering of their contents in the most advanced stage. Otherwise, stages I and II did not reveal the same profile for the different *Lactarius* species. Higher contents of bioactive compounds were found in stage I (immature fruiting bodies) for *L. deliciosus*, while for *L. piperatus*, stage II (mature with immature spores) presented the highest content. It seems that, for fruiting bodies without mature spores, the cap diameter is not correlated to the formation of bioactive compounds.

Table 5 shows the antimicrobial screening of *L. deliciosus* and *L. piperatus* in different stages of fruiting body maturity against *B. cereus*, *B. subtilis*, *S. aureus* (Gram+), *E. coli*, *P. aeruginosa*, *K. peumoniae* (Gram–) bacteria, and *C. albicans* and *C. neoformans* (fungi). The MICs for bacteria and fungi

Table 4. Bioactive Compound Contents of the Mushrooms in Different Stages of Maturity (Mean ± SD; $n = 3$)[a]

sample	total phenols (mg/g)	flavonoids (mg/g)	ascorbic acid (mg/g)	β-carotene (μg/g)	lycopene (μg/g)
L. deliciosus SI	5.84 ± 0.34 a	2.58 ± 0.09 a	0.11 ± 0.01 a	48.35 ± 0.40 a	32.63 ± 0.29 a
L. deliciosus SII	4.55 ± 0.24 b	2.20 ± 0.02 b	0.10 ± 0.00 b	32.37 ± 0.31 b	22.18 ± 0.22 b
L. deliciosus SIII	3.02 ± 0.11 c	1.84 ± 0.12 c	0.08 ± 0.00 c	26.20 ± 0.24 c	20.23 ± 0.17 c
L. piperatus SI (*38*)	5.52 ± 0.14 b	1.26 ± 0.09 b	0.15 ± 0.01 a	26.08 ± 0.05 b	8.14 ± 0.03 b
L. piperatus SII	5.76 ± 0.09 a	1.58 ± 0.02 a	0.16 ± 0.01 a	33.78 ± 0.05 a	13.04 ± 0.02 a
L. piperatus SIII	3.09 ± 0.12 c	0.35 ± 0.03 c	0.13 ± 0.01 b	17.22 ± 0.00 c	5.80 ± 0.01 c

[a] In each row and for each species, different letters mean significant differences ($p < 0.05$).

Table 5. Antimicrobial Activity of the Mushrooms in Different Stages of Maturity[a]

samples	MIC (mg/mL)							
	B. cereus	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*K. peumoniae*	*C. albicans*	*C. neoformans*
L. deliciosus SI	0.5 (+++)	0.5 (+++)	5 (++)	5 (++++)	50 (−)	50 (−)	50 (−)	50 (−)
L. deliciosus SII	50 (−)	5 (+++)	50 (+++)	5 (++++)	50 (−)	50 (−)	50 (−)	50 (−)
L. deliciosus SIII	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)
L. piperatus SI	50 (−)	50 (−)	0.5 (++++)	5 (++++)	50 (−)	5 (++)	50 (−)	50 (−)
L. piperatus SII	0.5 (++++)	0.5 (++++)	0.5 (++++)	0.5 (+++)	5 (+++)	0.5 (++)	50 (−)	50 (−)
L. piperatus SIII	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)	50 (−)
Ampicillin	0.00313 (++++)	0.0125 (++++)	0.00625 (++++)	0.00625 (++++)	0.00625 (++++)	0.00625 (++++)	NT	NT
Cycloheximide	NT	NT	NT	NT	NT	NT	0.0125 (++)	0.00625 (++++)

[a] No antimicrobial activity at the higher tested concentration (−), inhibition zone < 1 mm. Slight antimicrobial activity (+), inhibition zone 2–3 mm. Moderate antimicrobial activity (++), inhibition zone 4–5 mm. High antimicrobial activity (+++), inhibition zone 6–9 mm. Strong antimicrobial activity (++++), inhibition zone > 9 mm. Standard deviation ±0.5 mm. NT, not tested.

were determined as an evaluation of the antimicrobial activity of the tested mushrooms. The halos of the inhibition zones corresponding to the MICs are also presented. All the stages of maturity revealed antimicrobial activity showing different selectivity and MICs for each microorganism. The highest content of bioactive compounds in the first stages might account for the better results found in their antimicrobial activity. For *L. deliciosus*, stage I (immature) presented strong antimicrobial activity, while in *L. piperatus*, the most effective stage was stage II (mature with immature spores). Until mature spores are no longer present, the results seem to be independent of maturity evolution, depending only on the bioactive compound content present in each stage. A similar correlation between phenol content and antimicrobial properties was already observed by us in a previous work (*9*).

L. piperatus showed better results than *L. deliciosus* at the same stage of maturity (lower MICs), which is in agreement with the higher content of bioactive compounds found in the first species (**4**). Nevertheless, there might be other antimicrobial compounds besides those determined in this study. Fruiting bodies of pungent *Russulaceae* species (genera *Lactarius* and *Russula*) initially contain a biologically inactive fatty acid ester of velutinal (stearoylvelutinal). Upon injury (by insects or snails biting and chewing the fruit body), that compound is enzymatically transformed within seconds to pungent unsaturated dialdehydes and related sesquiterpenes. For instance, *L. piperatus* form velleral and piperdial. The mechanism of these conversions seems to constitute a chemical defense system that protects the fruit bodies of these species against parasites and fungivors (*37*).

The extracts inhibited all the tested Gram+ bacteria, showing no antifungal activity. Concerning Gram–bacteria, only *L. piperatus* revealed activity against the three species tested, with *E. coli* and *K. pneumoniae* resistant to *L. deliciosus* in all stages of maturity. As expected due to its lower content in bioactive compounds, the mature stage with mature spores (stage III) was the least effective, presenting no antimicrobial activity at 50 mg/mL. Usually, pure active compounds reveal more activity than crude extract, and as expected, the standards ampicillin (antibacterial) and cycloheximide (antifungal) presented lower MICs than the mushroom extracts.

Antimicrobial compounds with more or less strong activity could be isolated from many mushrooms and they could be beneficial for humans. Until now, only compounds from microscopic fungi are on the market as antibiotics, and therefore, it is important to screen the antimicrobial activity of mushrooms and draw conclusions regarding the best stage of maturity to harvest wild species. The knowledge about their chemical composition in different stages of fruiting body maturity will be also useful in order to find the best stage to achieve better functional and nutritional properties. In this study, we concluded that the last stage of fruiting body maturity is not recommended for those proposals.

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