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Bio compounds of edible mushrooms: *in vitro* antioxidant and antimicrobial activities

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ABSTRACT

The aim of this study was to optimize the extraction of total phenolics from edible mushrooms, evaluate the *in vitro* antioxidant and antimicrobial activities and identify the main phenolic compounds present in the extracts. A Box-Behnken design was used and the effects of temperature (X1, 25–55 °C), solvent-to-solid ratio (X2, 30–70 mL per gram) and solvent concentration (X3, 25–75%) were evaluated. In the optimum conditions of extraction, the antioxidant (DPPH, ABTS and FRAP assays) and antimicrobial activities of the extract were tested against the bacteria: *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis*. In addition, the phenolic compounds of the extracts were quantified. The *A. brasiliensis* mushrooms showed the higher phenolic contents (13.16 mgGAE/g) and antioxidant activity by DPPH and ABTS assays of 50.64 and 128.60 μmolTE/g, respectively, among the phenolic extracts of the mushrooms analyzed. The gallic acid was the main phenolic compound identified and the *A. brasiliensis* had the highest concentration (491.89 μg/g). All extracts presented antibacterial activity for Gram-positive strains (MIC ≤ 200 mg/mL). The high content of antioxidant compounds, extracted by a non-toxic solvent, suggested that the *A. brasiliensis* extract can be applied in the food industry as a natural antioxidant.

1. Introduction

Different species of mushrooms have been identified as a source of bioactive compounds, in addition to presenting an important nutritional value (Carneiro et al., 2013; Kalač, 2013). During their cultivation, mushrooms produce valuable secondary metabolites (for example, phenolic compounds) that have beneficial properties such as antioxidant, antimicrobial, anti-inflammatory, anti-mutagenic, anti-allergic and cardioprotective effects (Ahmad et al., 2014; Nedelkoska et al., 2013).

Phenolic compounds in mushrooms are excellent antioxidants and synergists, but are not mutagenic (Yildiz, Can, Laghari, Sahin, & Malkoç, 2015). Carneiro et al. (2013), Lin et al. (2014), Gasecka, Siwulski, and Mleczek (2017) among other researchers found several phenolic acids (gallic, ferulic, chlorogenic, caffeic, p-hydroxybenzoic, salicylic, sinapic, syringic, p-coumaric, vanilic, 2,5-dihydroxybenzoic, protocatechuic and t-cinnamic acid) while evaluating a few mushrooms, such as *Pleurotus, Agaricus, Lentinula, Armillaria, Auricularia, Fistulina* among others. The reported effects are mainly associated with the antioxidant properties of phenolic compounds due to redox reactions, which allow them to act as donors of hydrogen atoms or as reducing agents (Ahmad et al., 2014).

Mushrooms have been shown to have antibacterial activity due to the presence of molecules that make up their fruiting bodies and have different molecular weights (Alves et al., 2012; Erjavec et al., 2016). According to the bibliographic survey carried out by Alves et al. (2012), most of the mushroom extracts studied have higher antibacterial activity against gram-positive strains than against gram-negative strains.

Extracts containing antioxidant compounds have become a new source for the food industry in order to replace synthetic antioxidants and to complement industrialized products with bioactive compounds (Zielinski, Haminiuk, & Beta, 2016). The extraction of bioactive compounds from raw materials is the first step in the use of phytochemicals

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in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceuticals and cosmetics (Dai & Mumper, 2010). The most commonly used methods for extracting phenolic compounds are those that use solvents such as ethanol, acetone, methanol, or a mixture of these with water (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010). However, for food applications, phenolic extraction is preferentially carried out with ethanol as it is considered non-toxic, green, biocompatible and more economically viable (Ilaiyaraja, Likhith, Sharath Babu, & Khanum, 2015).

Extraction of the phenolic compounds may be influenced by different factors, such as temperature, time, solvent concentration and solvent-to-solid ratio, agitation, particle size, and pH, among others. The combination of these factors and the determination of optimal conditions are important in order to obtain a maximum extraction potential. Response surface methodology (RSM) is a useful tool for chemical and biochemical processes optimization. It is an effective mathematical technique for analyzing the relationships between response and independent variables, interactions among factors, as well as optimization factors that can influence process results (Lim & Yim, 2012).

No optimization study about the extraction process of bioactive compounds of edible mushrooms with the use of non-toxic solvents was found in the literature. Therefore, the main objectives of this study were: i) to optimize the extraction of phenolic compounds from five edible mushrooms by RSM; and ii) to evaluate the antioxidant and antimicrobial activity *in vitro* of the optimized extracts and determining the main individual phenolic compounds by UHPLC-DAD.

2. Material and methods

2.1. Preparation of samples

Five samples of edible mushrooms were analyzed during the years 2015 and 2016. A total of 2.0 kg of each mushroom (individual trays of 400 g), *Agaricus bisporus* (Champignon and Portobello), *Flammulina velutipes* (Enoki) and *Lentinula edodes* (Shiitake) were purchased at the local market in Curitiba, Paraná State. It is not common to commercialize the fresh *A. brasiliensis* owing to its high perishability and for this reason it was acquired directly on the farm of fresh mushrooms (from Piedade, São Paulo State, Brazil) and transported immediately to the laboratory for analyzes. The other mushrooms were purchased between the 1st and 3rd day after harvesting, considering the shelf life of 10 days. The samples were frozen, lyophilized at −50 °C and 150 mm Hg for 96 h (Liotop, modelo L101) and ground until the particles measured less than 32 mesh (0.5 mm). The powdered mushrooms were vacuum-packed and stored under light protection until analysis.

2.2. Optimization of the extraction of total phenolic compounds (TPC)

Optimization extraction parameters of TPC of each mushroom were performed using a Box-Behnken design (Box & Behnken, 1960). The complete design consisted of 15 experiments, including 3 replicates of the central point. The factors (independent variables) evaluated for extraction were: temperature (X1, 25–55 °C), solvent-to-solid ratio (X2, 30–70 mL per gram) and ethanol concentration (X3, 25–75%) (Table 1). Total phenolic compounds were the dependent variable. The extraction time was pre-established by means of preliminary tests (data not shown) in 2 h with continuous agitation of 100 rpm (Shaker New Brunswick Scientific, model I 26, GER.). Furthermore, the experiments were conducted randomly and in triplicate. The samples were then centrifuged at 1075.20 × *g* for 15 min (Daiki centrifuge, model 80-2B, CHN.) and the supernatant was analyzed.

2.3. Total phenolic compounds (TPC)

TPC of the extracts were determined using the Folin-Ciocalteu reagent, according to the procedure described by Singleton and Rossi (1965), with minor modifications. The absorbance was measured at 725 nm and the values obtained were compared with a calibration curve of gallic acid (0–100 mg/L). The results were expressed in mg gallic acid equivalent (GAE) per gram of dry mushrooms (mg GAE/g dm). All determinations were performed in three replicates.

2.4. In vitro antioxidant assays (DPPH, ABTS and FRAP)

The free radical scavenging through the DPPH assay was determined according to Brand-Williams, Cuvelier, and Berset (1995), with minor modifications. First, 0.1 mL of the extract (obtained in section 2.2) was added to 3.9 mL of the DPPH methanolic solution (0.06 mmol/L). The mixture was allowed to react in the dark for 30 min and the absorbance was then measured at 515 nm.

The free radical scavenging by ABTS radical was determined according to Re et al. (1999). A volume of 88 µL of potassium persulfate (140 mmol/L) was added to 5 mL of ABTS (7 mmol/L). The mixture was stored in an amber bottle in the dark and at room temperature for 16 h. The ABTS solution absorbance was adjusted at 0.70 ± 0.05 at the 734 nm in spectrophotometer. Then, 30 µL of the optimized hydroethanolic extract of the edible mushrooms were added to a 3 mL ABTS solution. The mixture remained in the dark for 2 h at room temperature and the absorbance was measured at 734 nm.

The ferric reducing antioxidant power assay (FRAP) was determined according to the methodology described by Benzie and Strain (1996), with minor modifications. The FRAP reagent was prepared by a mixture of acetate buffer (300 mmol/L, pH 3.6), TPTZ (10 mmol/L) solubilized in HCl (40 mmol/L) and ferric chloride (20 mmol/L), in the ratio 10/1/1 (v/v/v), respectively. Then, 3 mL of the FRAP reagent was added to 0.1 mL of ethanolic extract from each mushroom. The mixture was kept at room temperature in the dark. After 30 min, the absorbance was measured at 593 nm.

All antioxidant activities assays were performed in three replicates and measurement was performed in a spectrophotometer UV/VIS (Shimadzu, model 1800, Kioto, JPN). The results were compared with a standard curve (Trolox 0–2500 µmol/L) and expressed in µmol Trolox equivalent per gram of dry mushroom (TE µmol/g dm).

2.5. Ultra high-performance liquid chromatography (UHPLC-DAD)

Phenolic and non-phenolic compounds were determined according to the method proposed by Gasic et al. (2014), with slight changes. Initially, the mushroom extracts were filtered through a 0.22 µm nylon syringe filter and 5 µL of the sample was injected into an UHPLC H-Class (Waters, Milford, MA, USA), equipped with a Waters quaternary pump system. An auto-sampler (Milford, MA, USA) and a diode array detector (Waters, Milford, MA, USA) were used. An Acquity BEH C18 (50 mm × 2.1 mm) column with 1.7 µm particles (Waters, Milford, MA, USA) was used at 30 °C.

The mobile phase A consisted of a system solvent water/formic acid (99.9/0.1 v/v), while the mobile phase B used methanol/formic acid (99.9/0.1, v/v) with flow of 5 mL/min. The linear gradient was programmed as follows: 0–8 min, 0–20% B; 8–15 min, 20–100% B; 15–18 min 100-0% B, followed by isocratic elution with 0% B until 20 min. Monitoring of the chromatograms was performed at 280, 290 and 370 nm, since most phenolic compounds exhibit maximum absorptions near these wavelengths. The quantification was performed using calibration curves of standards of gallic acid, protocatechuic acid, 1,2-dihydroxybenzene, gentisic acid, *p*-hydroxybenzoic acid, trans-cinnamic acid, *p*-coumaric acid, ferulic acid, vanillin, fumaric acid and benzoic acid.

2.6. In vitro antimicrobial activity

2.6.1. Microorganisms tested and preparation of the bacterial suspension

Antimicrobial activities of optimized mushroom extracts were tested against 4 bacteria: 2 Gram-positive (*Staphylococcus aureus* ATCC

Table 1
Total phenolic compounds obtained by Box-Behnken planning.

Assay	Temperature (°C)	Solvent-to-solid ratio (mL/g)	Solvent concentration (%)	Total Phenolic Compounds mg GAE/g[a]				
				A bisporus (Champignon)	*A bisporus* (Portobello)	*A. brasiliensis*	*F. velutipes*	*L. edodes*
1	25	30	50	5.80 ± 0.04	5.88 ± 0.04	9.45 ± 0.10	5.78 ± 0.03	3.91 ± 0.05
2	55	30	50	7.42 ± 0.12	7.78 ± 0.05	9.86 ± 0.07	2.04 ± 0.02	3.88 ± 0.04
3	25	70	50	5.70 ± 0.06	9.74 ± 0.12	11.38 ± 0.19	6.42 ± 0.06	4.36 ± 0.08
4	55	70	50	8.17 ± 0.14	9.25 ± 0.02	13.22 ± 0.10	5.17 ± 0.06	4.40 ± 0.04
5	25	50	25	6.35 ± 0.03	4.89 ± 0.04	10.00 ± 0.06	7.52 ± 0.05	5.59 ± 0.05
6	55	50	25	6.19 ± 0.10	6.63 ± 0.03	9.38 ± 0.08	5.52 ± 0.08	3.95 ± 0.05
7	25	50	75	7.20 ± 0.07	8.55 ± 0.00	11.42 ± 0.34	5.79 ± 0.01	3.69 ± 0.07
8	55	50	75	8.51 ± 0.16	9.67 ± 0.09	12.13 ± 0.26	4.56 ± 0.05	3.78 ± 0.02
9	40	30	25	4.81 ± 0.02	5.49 ± 0.01	8.39 ± 0.09	6.35 ± 0.17	4.66 ± 0.00
10	40	70	25	6.31 ± 0.08	6.71 ± 0.04	10.70 ± 0.56	6.34 ± 0.03	5.19 ± 0.015
11	40	30	75	7.16 ± 0.10	8.08 ± 0.05	9.86 ± 0.21	6.09 ± 0.03	3.29 ± 0.03
12	40	70	75	8.09 ± 0.13	9.60 ± 0.02	12.66 ± 0.12	6.24 ± 0.04	3.74 ± 0.11
13 (C)	40	50	50	7.04 ± 0.15	7.93 ± 0.04	11.50 ± 0.14	6.01 ± 0.04	4.03 ± 0.04
14 (C)	40	50	50	7.24 ± 0.08	7.93 ± 0.14	11.68 ± 0.42	6.24 ± 0.04	4.21 ± 0.04
15 (C)	40	50	50	6.94 ± 0.07	8.09 ± 0.05	11.62 ± 0.15	6.15 ± 0.06	4.12 ± 0.02

[a] GAE – Gallic acid equivalent. (C) Central point.

25923 and *Bacillus cereus* ATCC 11778) and 2 Gram-negative (*Escherichia coli* ATCC 25922 and *Salmonella enteritidis* ATCC 13076). The microorganisms were supplied by the Microbiology Laboratory of the Federal Technological University of Paraná, Campus of Curitiba (*E. coli* and *S. aureus*), Department of Biochemistry of the Federal University of Paraná (*B. cereus*) and Enterobacteria Laboratory of Oswaldo Cruz Institute (*S. enteritidis*).

Bacterial suspensions were prepared according to the protocol described by Wiegand, Hilpert, and Hancock (2008). The turbidity of the initial suspension was adjusted by comparison with the 0.5 McFarland standard. The initial suspension contained about 10^8 colony forming units (CFU)/mL. Subsequently, dilutions were prepared in 0.9% saline, 1:100 from the initial bacterial suspension.

2.6.2. Microdilution method

The antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) using the microdilution method according to the protocol described by Wiegand et al. (2008). The optimized extracts (obtained in section 2.2) were lyophilized and rehydrated with sterile water.

The 96-well plates were prepared by distributing of 50 µL of Mueller-Hinton nutrient broth (MH) for bacteria. A volume of 50 µL was added from the phenolic extract solution of each mushrooms (concentration = 400 mg/mL) in the first row of the plate and serial (1:1) extract dilutions in MH broth were made. The concentration range obtained for the extracts was 200 to 0.39 mg/mL. Then, 50 µL of the bacterial suspension in MH broth (about 10^5 CFU/mL) was added to the wells content. The microplates were incubated at 37 °C for 18–20 h.

The MIC was defined as the lowest concentration of the tested extracts that prevented microbial growth. 2,3,5-Triphenyltetrazolium chloride was used to evaluate the change in the color of the medium (colorless to pink), indicating bacterial growth. Each test plate included growth control and sterility control. The antibiotic used as a reference was amoxicillin (initial concentration 64 mg/L). It was effective in inhibiting all the bacteria tested in the following concentrations: 4 mg/L (*B. cereus*), 0.25 mg/L (*S. aureus*), 1 mg/L (*S. enteritidis*) and 12.50 mg/L (*E. coli*). All determinations were made in triplicates.

2.7. Data analysis

All data were showed were presented as mean ± standard deviation (SD). In order to model the extraction of total phenolic antioxidants from mushrooms, RSM coupled with multiple linear regression was used. Then, a second-order polynomial model was used to express TPC extraction, as a function of independent variables, according to equation (1).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

Where: Y is the expected response, β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for the intercept terms, linear, quadratic and interaction, respectively. X_i, and X_j are the levels of the independent variables (Bruns, Scarmino, & Barros Neto, 2006).

The optimized conditions of the models proposed were experimentally tested in order to validate the models and to verify their predictive power when comparing the predicted theoretical data with the experimental data (Annex 1). After obtaining the best extraction conditions of phenolic compounds, new extracts were obtained in the best conditions. The objectives were of determining the antioxidant activity using the DPPH, ABTS and FRAP methods, the phenolic profile and the antimicrobial activity. The data were also submitted for the analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$ was considered significant). All analyses were performed using the software Statistica 8.0 (Statsoft Inc., Tulsa. Okla., U.S.A.) and had a significance level of $p \leq 0.05$.

3. Results and discussion

3.1. Optimization of the extraction

An optimization on the basis of the total phenolic compounds was performed for each mushroom (Table 1 and Supplementary material). All models determined by multiple regression analysis showed significance ($p < 0.01$) but did not present a lack of fit ($p > 0.05$). The adequacy of the models was higher than 93.8% of all variance in the data with an adjusted $R^2 > 0.893$. Therefore, the influence of each effect on the extraction ranged according to mushroom used. For the *Agaricus* species, all the linear coefficients [temperature (X1), solvent-to-solid ratio (X2), and solvent concentration (X3)] evaluated had a significantly ($p < 0.05$) positive effect, increasing the extraction of the phenolic compounds. The mathematical model obtained for *A. bisporus* suggested that the solvent concentration was the linear coefficient that contributed the most in the recovery of the total phenolics, while that in *A. brasiliensis* was the solvent-to-solid ratio followed by solvent concentration. On the other hand, for the *Lentinula* and *Flammulina* species, temperature (X1) and solvent concentration (X3) showed a significant negative effect on phenolic extraction. In other words, these effects provided a decrease in the extraction of total phenolic compounds. The

Table 2
Total phenolic compound values (experimental and predicted by the equation) of the extracts obtained under the optimum extraction conditions.

Mushrooms	Optimal conditions for the extraction of TPC[a]			Total Phenolic Compounds (mg GAE/g dm)[b]			
	Temperature (°C)	Solvent to solid ratio (mL/g)	Solvent concentration (%)	Observed	Predict	−95% Pred	+95% Pred
A. bisporus (Champignon)	55	70	75	9.53^{c} ± 0.16	9.37	8.33	10.42
A. bisporus (Portobello)	55	70	75	9.97^{b} ± 0.21	10.24	9.35	11.13
A. brasiliensis	55	70	75	13.16^{a} ± 0.06	13.50	12.48	14.53
F. velutipes	25	60.34	25	8.38^{d} ± 0.13	7.58	6.43	8.73
L. edodes	25	59	25	5.66^{e} ± 0.10	5.60	5.41	5.77

Means followed by the same letter in the column do not differ significantly as per Tukey's test ($p \leq 0.05$).
[a] TPC – Total phenolic compounds.
[b] GAE – Gallic acid equivalent.

quadratic regression coefficients of solvent-to-solid ratio (X2) showed a significantly negative effect in the extraction of phenolics for *A. bisporus* (Champignon), while for *A. bisporus* (Portobello) the quadratic solvent concentration (X3) coefficient contributed negatively to the extraction process. In *A. brasiliensis* both quadratic coefficients (X2 and X3) had a significantly negative effect. The opposing behavior was observed in the quadratic solvent concentration (X3) coefficients of the models of *F. velutipes* and *L. edodes*. Lastly, the interaction (linear x linear and linear x quadratic) coefficients had a different influence for each mushroom extracted, showing positive or negative effect according to the model proposed (Supplementary material).

After modeling the extraction, the optimum point was determined using desirability function (d = 1.00). The optimal conditions for the TPC extraction are presented in Table 2. The optimum temperature found for the extraction of the phenolic compounds from *Agaricus* genus mushrooms was 55 °C, whereas for *L. edodes* and *F. velutipes* it was 25 °C. The higher proportion of solvents-to-solids were the ones that had highest yield in the extraction of the TPC, as suggested by the positive coefficients of the linear effect X2 (solvent-to-solid ratio) of the models proposed (supplementary material). *Agaricus* genus extracts presented the higher TPC yield when the solvent-to-solid ratio was 70 mg/mL.

The optimum conditions (Table 2) of TPC extraction of the mushrooms of the genus *Agaricus* suggest that the solvent used must be at the concentration of 75% (ethanol: water), in order to obtain the best yields of TPC. In contrast, the best yields for the TPC extraction of the *F. velutipes* and *L. edodes* mushrooms were obtained with the solvent in the concentration of 25% (ethanol: water). It is possible that the less hydrophilic solvent (75% ethanol) facilitated the extraction of TPC from the matrix of the fungi of the genus *Agaricus* due to its greater interaction and permeability to the hydrophobic groups present in the dehydrated material. According to studies by Bach, Helm, Bellettini, Maciel, and Haminiuk (2017), the genus *Agaricus* presents higher lipid and protein content when compared to the mushrooms *F. velutipes* and *L. edodes*. In addition, the amino acids that make up for the *Agaricus* proteins have higher amino acid content with the apolar groups.

On the other hand, the solvent with the lowest proportion of ethanol (25%) was more effective in extracting TPC from the *F. velutipes* and *L. edodes* mushrooms, which had lower proportions of hydrophobic compounds in their physicochemical composition (Bach et al., 2017). According to Zielinski et al. (2016) mixtures between water and alcohol have been more efficient in the extraction of phenolic compounds when compared to the monocomponent solvent system. Methanol and ethanol are the main kinds of alcohol used in the extraction; however ethanol has advantages due to its safety for human consumption.

3.2. Phenolics and antioxidant power

Phenolic compounds are hydroxylated aromatic compounds with one or more aromatic rings, and one or more hydroxyl groups. These hydroxyls are responsible for the antioxidant properties of these compounds, since they have the ability of eliminating free radicals (Liu, Jia, Kan, & Jin, 2013).

TPC concentration for the five evaluated mushrooms ranged from 5.66 to 13.16 mg GAE/g dm. *A. brasiliensis* presented the highest level of phenolic compounds ($p \leq 0.05$), while the lowest was found for *L. edodes*. Keleş, Koca, and Gençcelep (2011) quantified the TPC of 24 mushrooms and found values ranging from 0.42 to 12.78 mg GAE/g.

The antioxidant activities of the mushrooms were evaluated by the DPPH, ABTS and FRAP methods (Table 3). The DPPH assay ranged from 13.12 to 50.64 μmol TE/g; the ABTS from 34.57 to 128.60 μmol TE/g and the FRAP from 14.66 to 48.26 μmol TE/g dm. The extract of *A. brasiliensis* presented a higher level of antioxidant activity regarding free radical scavenging evaluated by DPPH and ABTS methods, whereas *A. bisporus* presented the highest level for FRAP. Using Pearson's correlation (for the data of Tables 2 and 3), it can be observed that TPC shows a strong correlation with the antioxidant activity measured by the ABTS (r = 0.973), DPPH (r = 0.828) and FRAP (r = 0.607) methods, indicating that the TPC content has a direct relationship with antioxidant activity of mushroom hydroethanolic extracts.

Although the extracts evaluated in each assay were the same, the action mechanisms involved in the antioxidant tests are different; however, only the antioxidant assays FRAP x ABTS (Table 3) showed no significant correlation (r > 0.64 p > 0.05). DPPH X FRAP and DPPH X ABTS tests showed a significant correlation (r > 0.908, p < 0.05) and (r > 0,887, p < 0.05), respectively. The free-radical cations DPPH and ABTS are two stable and colored free radicals, receptors for both a hydrogen atom and an electron to become a stable diamagnetic molecule. Upon receiving a hydrogen atom or an electron from an antioxidant agent, such as phenolic compounds, the reduced form of the radical is generated, followed by a loss of color (Mujic, Zekovic, Lepojevic, Vidovic, & Zivkovic, 2010; Zielinski et al., 2016). Meanwhile, FRAP is characterized only by the electron transfer ability, which results in the reduction of iron ions (Fe^{3+} to Fe^{2+}) in the presence of

Table 3
Antioxidant activity of the extracts obtained under the optimum extraction conditions.

Mushrooms	Antioxidant activity (μmol TE/g)[a]		
	DPPH[b]	ABTS[c]	FRAP[d]
A. bisporus (Champignon)	34.35^{c} ± 0.21	74.06^{b} ± 1.73	40.84^{c} ± 0.63
A. bisporus (Portobello)	40.00^{b} ± 0.40	74.41^{b} ± 2.23	48.26^{a} ± 0.15
A. brasiliensis	50.64^{a} ± 0.37	128.60^{a} ± 2.02	43.25^{b} ± 0.26
F. velutipes	13.12^{e} ± 0.20	50.44^{c} ± 1.48	14.66^{e} ± 0.65
L. edodes	21.44^{d} ± 0.51	34.57^{d} ± 2.77	26.77^{d} ± 0.16

Means followed by the same letter in the column do not differ significantly as per Tukey's test ($p \leq 0.05$).
[a] TE – Trolox equivalent.
[b] 2,2-diphenyl-1-picryl-hydrazyl.
[c] 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid).
[d] Ferric reducing antioxidant power.

Table 4
Phenolic and non-phenolic compounds determined by UHPLC-DAD in five edible mushrooms.

Analyzed compounds		*A. bisporus* (Champignon)	*A. bisporus* (Portobello)	*A. brasiliensis*	*F. velutipes*	*L. edodes*
Phenolic Compounds (μg/g dry matter)	1	$259.54^{c} \pm 3.20$	$282.97^{b} \pm 0.11$	$491.89^{a} \pm 1.59$	nd	$148.92^{d} \pm 3.09$
	2	nd	nd	nd	$10,89 \pm 0.17$	nd
	3	$236.35^{a} \pm 1.33$	$159.73^{b} \pm 1.05$	$148.83^{c} \pm 0.81$	$97.55^{d} \pm 1.51$	nd
	4	nd	nd	27.73 ± 1.10	nd	nd
	5	$9.86^{c} \pm 0.65$	$57.12^{b} \pm 1.20$	$332.76^{a} \pm 2.98$	nd	$11.56^{c} \pm 0.42$
	6	nd	nd	$11.14^{a} \pm 0.50$	nd	$4.15^{b} \pm 0.02$
	7	nd	nd	24.47 ± 0.64	nd	nd
	8	nd	nd	752.54 ± 1.90	nd	nd
	9	nd	nd	nd	nd	1.56 ± 0.06
	Total phenolic compounds	505.75	499.82	1789.36	108.44	166.19
Non Phenolic Compounds (μg/g dry matter)	10	$2824.05^{b} \pm 14.10$	$2475.41^{c} \pm 19.56$	$3232.50^{a} \pm 30.90$	$2184.54^{d} \pm 5.34$	$1301.76^{e} \pm 2.29$
	11	$215.34^{ab} \pm 0.84$	$220.57^{a} \pm 3.19$	$204.99^{b} \pm 1.05$	$61.09^{d} \pm 0.84$	$91.74^{c} \pm 7.84$
	Total non-phenolic compounds	3039.39	2695.98	3437.49	2245.63	1393.50

1 - Gallic acid (3,4,5-trihydroxybenzoic acid), 2 – Protocatechuic acid (3,4-dihydroxybenzoic acid), 3 – Catechol (1,2-dihydroxybenzene), 4 - Gentisic acid (2,5-dihydroxybenzoic acid), 5 - p-Hydroxybenzoic acid (4-hydroxybenzoic acid), 6 - Trans-cinnamic acid, 7 - p-Coumaric acid (4-hydroxycinnamic acid), 8 - Ferulic acid, 9 – Vanillin (4-hidroxi-3-metoxi-benzaldeído), 10 – Fumaric acid. 11 - Benzoic acid. nd – not detected. Means followed by the same letter in the line do not differ significantly as per Tukey's test ($p \leq 0.05$).

antioxidant compounds (Craft, Kerrihard, Amarowicz, & Pegg, 2012).

Phenolic acids are classified as hydroxybenzoic and hydroxycinnamic (Dai & Mumper, 2010). Among the hydroxybenzoic acids determined in mushrooms (Table 4) are gallic, *p*-hydroxybenzoic, protocatechuic and gentisic. The phenolic acids derived from cinnamic acid identified were trans-cinnamic, *p*-coumaric and ferulic acid.

Several researchers have identified one or more phenolic compounds in many species of mushrooms (Gasecka et al., 2017; Liu et al., 2013; Mattila et al., 2001; Reis, Barros, Martins, & Ferreira, 2012; Taofiq et al., 2016). Liu et al. (2013) determined 280 μg/g of gallic acid in *A. bisporus*, while Carvajal et al. (2012) found 1910 μg/g of gallic acid for *A. brasiliensis*. In the present study 259.54 and 491.89 μg/g of acid gallic for *A. bisporus* and *A. brasiliensis*, respectively, were found.

Mattila et al. (2001) studied the mushrooms *A. bisporus* (Champignon and Portobello) and *L. edodes*. The authors quantified trans-cinnamic acid, ranging from 1.47 to 2.69 μg/g; p-hydroxybenzoic acid from 0.51 to 7.90 μg/g and protocatechuic acid from < 0.30 to 1.39 μg/g. These results reported are in accordance with the results obtained in this research (Table 4).

Taofiq et al. (2016) and Barros, Dueñas, Ferreira, Baptista, and Santos-Buelga (2009) determined the p-hydroxybenzoic acid in *L. edodes* (83.05 μg/g) and *A. bisporus* (25.58 μg/g), respectively. The p-hydroxybenzoic acid contents cited were higher than those found in our work for the same species: 11.56 μg/g in *L. edodes* and 9.86 μg/g in *A. bisporus*. In a way similar to our research, Barros et al. (2009) and Reis et al. (2012) also did not identify protocatechuic acid in *A. bisporus*. Reis et al. (2012) did not find p-coumaric acid in *A. bisporus* (Portobello) and *L. edodes*; however they determined the same in *A. bisporus* (2.31 μg/g).

The main phenolic acid identified was gallic acid, which showed a significant correlation ($p < 0.05$) with TPC (r = 0.95) and antioxidant activity methods [DPPH (r = 0.96) and ABTS (r = 0.99)]. The highest level of gallic acid was obtained from *A. brasiliensis*. Gallic acid is a planar molecule, consisting of an aromatic ring, three phenolic hydroxyl groups and one carboxylic acid group, providing the antioxidant activity of this molecule (Badhani, Sharma, & Kakkar, 2015). Galato et al. (2001) demonstrated, through the study of eight phenolic compounds and analogues that the antioxidant activity of a molecule increases with a growing number of hydroxyl groups attached to its aromatic ring. Other factors, such as the number and position of the hydroxyl group, the presence of other functional groups and their position in relation to the hydroxyl groups affect antioxidant and anti-radical activity. It was found that gallic acid exhibited the greatest antioxidant capacity among the various polyphenols (Badhani et al., 2015).

Another benzoic acid derivative that showed an important correlation with antioxidant and TPC methods was p-hydroxybenzoic acid [TPC (r = 0.82), DPPH (r = 0.83), ABTS (r = 0.90)]. Its antioxidant activity stems from the hydroxyl position in the molecule, which presents two methoxy groups adjacent to the OH group, substantially increasing the availability of hydrogen for reaction (Rice-Evans, Miller, & Paganga, 1996). The fumaric and benzoic acids were detected in all the analyzed mushrooms. Carvajal et al. (2012) also identified the acids fumaric and benzoic in *A. brasiliensis*.

Natural extracts may be more efficient than isolated bioactive compounds, since the synergistic interaction of the compounds may enhance the bioactive properties of the individual components. In addition, the use of natural extracts may be beneficial considering that the legal maximum levels regarding synthetic food additives are established based on several toxicological parameters that are generally not applicable to naturally occurring compounds (Oliveira, Angonese, Gomes, & Ferreira, 2016).

3.3. In vitro antimicrobial activity

Table 5 shows the minimum inhibitory concentration (MIC) of the optimized phenolic extract from five edible mushrooms against Gram-

Table 5
Antimicrobial activity for the optimized extracts from edible mushrooms, evaluated by the microdilution method MIC (mg/mL).[a]

Micro organisms tested	Gram-positive		Gram-negative	
	B. cereus	*S. aureus*	*S. enteritidis*	*E. coli*
A. bisporus (Champignon)	100	100	100	200
A. bisporus (Portobello)	50.00	200	200	200
A. brasiliensis	50.00	100	200	200
F. velutipes	25.00	50.00	-	-
L. edodes	12.50	1.56	100	100

[a] MIC – minimal inhibitory concentration; (-) MIC not found for the concentrations tested.

positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and Gram-negative bacteria (*Salmonella enteritidis* and *Escherichia coli*). The effectiveness of phenolic extracts was higher for Gram-positive microorganisms. This fact was also observed by Oyetayo (2009) while studying the antibacterial activity of *L. subnudus* against *B. cereus*, *S. aureus* and *S. typhinurium* bacteria. The *L. edodes* extract was more efficient for all tested bacteria, with an MIC of 1.56 mg/mL for *S. aureus*, similar to the findings of Taofiq et al. (2016) (MIC = 2.5 mg/mL). Nedelkoska et al. (2013) obtained an MIC ranging from 5 to 50 mg/mL while evaluating the antibacterial activity of five mushrooms.

It is possible that the higher resistance found in Gram-negative bacteria to antimicrobial agents is related to their sophisticated permeability barrier compared to the simpler cell membrane of Gram-positive bacteria. The cell membrane of Gram-negative species has an additional external lipopolysaccharide barrier that restricts the penetration of most molecules while being permeable to nutrients. This efficient permeability barrier has been blamed for the inability of the pharmaceutical industry to produce new classes of broad-spectrum compounds that are equally active against Gram-negative and Gram-positive bacteria (Oliveira et al., 2016).

With the exception of *F. velutipes*, the other mushrooms presented antibacterial activity regarding the four strains tested (Table 5). This mushroom showed the lowest antioxidant activity determined by the DPPH and ABTS tests, in addition to having the lowest concentration and variety of phenolic compounds identified. Such evidences may be related to its low antibacterial activity. The phenolic extract of *F. velutipes* did not inhibit Gram-negative bacteria (*S. enteritidis* and *E. coli*) for the tested concentrations (up to 200 mg/mL). Nedelkoska et al. (2013) found that the methanolic extract (up to 50 mg/mL) of *F. velutipes* also did not inhibit *E. coli*.

The most important finding refers to the fact that *F. velutipes* was the only mushroom that did not contain the phenolic acids: gallic and p-hydroxybenzoic. These phenols are some of the potential antibacterial agents present in mushrooms. According to Borges, Ferreira, Saavedra, and Simões (2013), gallic acid produces irreversible changes in the properties of the bacterial membrane through hydrophobicity changes, decreasing the negative surface charge as well as a rupture or pore formation in the cell membranes with consequent leakage of essential intracellular constituents. These conclusions were obtained after evaluation of the action mechanism of gallic acid in *S. aureus*, *E. coli*, *L. monocytogenes* and *P. aeruginosa*. Manuja, Sachdeva, Jain, and Chaudhary (2013) state that *p*-hydroxybenzoic acid (4-hydroxybenzoic acid) is indicated as an agent having antimicrobial activity against several bacteria, both Gram-positive and Gram-negative.

4. Conclusions

Among all the species of mushrooms studied, the *A. brasiliensis* showed the higher phenolic contents, antioxidant activity by DPPH and ABTS assays and content of gallic acid. In addition, it showed antimicrobial activity against the Gram-positive and Gram-negative strains. The high content of antioxidants was extracted by a non-toxic solvent, suggesting that the *A. brasiliensis* extract can be applied in the food industry as a natural antioxidant and antimicrobial agent.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2019.03.017.

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