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In vitro digestion of spermidine and amino acids in fresh and processed *Agaricus bisporus* mushroom

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ABSTRACT

Mushrooms are significant sources of amino acids and bioactive amines; however, their bioaccessibility can be affected by processing and during *in vitro* digestion. Fresh *Agaricus bisporus* mushroom was submitted to cooking and canning and samples were submitted to *in vitro* gastric and gastric-intestinal digestions. An UHPLC method was used for the simultaneous determination of 18 free amino acids, 10 biogenic amines and ammonia in the samples. Fresh mushroom contained 14 free amino acids, with predominance of alanine and glutamic acid; spermidine was the only amine detected; and ammonia was also detected. Spermidine levels were not affected by cooking, but there was a significant loss (14%) after canning. Spermidine levels were not affected by the *in vitro* gastric and intestinal digestion, suggesting full bioaccessibility. There was a significant decrease on total amino acids levels after cooking and canning, with higher losses for aspartic and glutamic acids in cooked and for aspartic acid and valine in canned mushrooms. After the *in vitro* gastric and intestinal digestions, the total levels of amino acids increased and two additional amino acids (arginine and methionine) were detected. During *in vitro* digestion many of the amino acids were released mainly in the intestinal phase. After *in vitro* digestion, amino acids per gram of protein of mushrooms are adequate for most FAO amino acid pattern for adults. Multivariate analysis confirmed that protein hydrolysis in processed mushrooms is higher in intestinal phase. Bioaccessibility data for spermidine in *A. bisporus* is a novelty and increase the value of this food.

1. Introduction

The consumption of edible mushrooms has increased in the last years worldwide. According to the Food and Agriculture Organization (FAO) of the United Nations, mushroom production was 6.9 million tons in 2008 and it increased to 8.9 million tons in 2018, representing a growth of more than 30.4% in ten years (FAOSTAT. Food and Agriculture Organization of the United Nations, 2020). In addition to the economic value, mushrooms have a small environmental footprint, as they grow from agricultural and forest wastes, requiring relatively little water and energy inputs, and generating low CO_2 emissions (AMI, 2020). Several mushroom species are commercially available; however, *Agaricus bisporus* represents 15% of the world's global mushroom supply (Royse, Baars, & Tan, 2017).

The occurrence and levels of bioactive amines in cultivated mushrooms have been described in the literature (Dadáková, Nova, & Kalač, 2009; Reis, Custódio, Botelho, Guidi, & Glória, 2020). High spermidine contents were detected in every mushroom, whereas agmatine, putrescine, tyramine, tryptamine and phenylethylamine were present depending on the type (Dadáková et al., 2009; Reis et al., 2020). Spermidine is an natural polyamine and its presence in mushroom is relevant as it plays several physiological functions, including cell division and proliferation, DNA and protein synthesis, regulation of apoptosis and prevention of oxidative stress (Agostinelli, 2020; Lenis, Elmetwally, Maldonado-Estradam, & Bazer, 2017). It is also effective in wound healing, modulation of the permeability and renewal of the intestinal mucosa, affecting the uptake of nutrients and allergenic proteins (Gloria, 2006; Kalač & Krausová, 2005; Kalač, 2014).

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Furthermore, it displays anti-aging effects due to its capacity to induce cytoprotective autophagy (Madeo, Eisenberg, Pietrocola, & Kroemer, 2018).

High levels of free amino acids are also found in commercial mushrooms. Amino acids are important food components because of their role in human health. They are the most important building blocks of body tissues, enzymes and hormones, so they are indispensable for vital functions (FAO/WHO, 2013; Levesque, Moehn, Pencharz, & Ball, 2010; Marchini, Vannucchi, Suen, & Cunha, 2016). In addition, the free amino acids profile of mushroom can affect its sensory properties. For example, aspartic and glutamic acids are umami, monosodium glutamate-like (MSG-like) or palatable taste amino acids. Some amino acids impart a sweet taste, e.g. alanine, glycine, proline, serine and threonine, whereas others are considered bitter amino acids, among them arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine. Lysine and tyrosine are tasteless amino acids (Chen et al., 2015; Chiang, Yen, & Mau, 2006; Ming, Li, Huo, & Chen, 2014; Rotola-Pukkila, Yang, & Hopia, 2019).

Mushrooms are very perishable food; therefore, processing can be used to improve shelf life (Liu et al., 2014; Pei et al., 2014). Heat treatment is an important unitary operation for food preservation, which includes, among others, pasteurization and sterilization methods such as steaming, boiling, roasting, microwave and others. These treatments can be applied to prepare the product, to develop desired flavors, color and aroma components, to modify the structure, to preserve or sterilize food through inactivation of microorganisms, toxins and enzymes by heat. However, despite increased food stability and safety with heat treatments, native compounds and nutrients, which are essential to the diet, can be lost, or have its bioavailability affected (Ariza et al., 2018; Cilla, Bosch, Barberá, & Alegría, 2018; Mercadante & Mariutti, 2018). In fact, food processing is one of the most important determinants of nutrient bioavailability, as it may have positive or negative impacts, affecting the bioaccessibility of nutrients and bioactive compounds (Mercadante & Mariutti, 2018). In mushroom, there can be losses due to lixiviation of water-soluble components and to Maillard browning from the reaction of amino acids and amines with carbonyls (Li et al., 2011; Rotola-Pukkila et al., 2019). Furthermore, during processing, there can be changes in the food matrix which can affect the bioaccessibility of these compounds.

Scarce information is available regarding the changes occurring on bioactive amines during processing and the influence of processing on the *in vitro* bioaccessibility of bioactive amines and amino acids in mushroom. In this context, the objective of this study was to investigate the *in vitro* bioaccessibility of free amino acids and bioactive amines in fresh and processed *Agaricus bisporus* mushroom. Mushroom was obtained from the market, submitted to cooking and canning. The fresh, cooked and canned products were subjected to gastric and intestinal *in vitro* digestions. The samples were analyzed for 18 free amino acids, 10 bioactive amines and ammonia simultaneously by UPLC. The results were compared to the amino acids requirements for adult. Samples were also clustered by principal components and hierarquical cluster analyses with respect to the profile and levels of amino acids.

2. Material and methods

2.1. Sample and reagents

Fresh *Agaricus bisporus* mushrooms (5 kg) were purchased at commercial maturity from distributors at Belo Horizonte, MG, Brazil. Bile salts (Sigma B-8756), pancreatin from porcine gastric mucosa (Sigma P-3292), pepsin from porcine gastric mucosa (Sigma P-7012), and standards for bioactive amines (spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine, serotonin hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride), L-amino acids (alanine, arginine hydrochloride, aspartic acid, cystine, glycine, glutamic acid, histidine hydrochloride, isoleucine, leucine, lysine hydrochloride, methionine, phenylalanine proline, serine, threonine, tryptophan, tyrosine, valine, and norvaline - internal standard), and ammonium chloride were from Sigma Chemical Co. (St. Louis, MO, USA). AccQ.FluorTM pre-column derivatization kit was purchased from Waters (Milford, MA, USA).

The reagents were of analytical grade, except acetonitrile, which was LC grade. Ultrapure water was obtained from a Milli-QTM system (Millipore Corp., Milford, MA, USA). The organic and aqueous solvents for the UHPLC analysis were filtered through 0.22 μm pore size HAWP and HVWP membranes, respectively (Millipore Corp., Milford, MA, USA).

2.2. Mushroom processing

Fresh mushrooms were divided into three sub lots – fresh, cooked and canned mushrooms. Fresh mushrooms were analyzed immediately for free amino acids and bioactive amines. Cooked mushrooms were prepared by heat treatment of 100 g fresh mushrooms in 1 L boiling water (97 °C) for 10 min (Soler-Rivas, Ramírez-Anguiano, Reglero, & Santoyo, 2009). To prepare canned mushrooms, the mushrooms were washed, allowed to soak in 1% sodium bisulfite for 2 min and heat treated in boiling water (97 °C) for 5 min. The blanched mushrooms (100 g) were drained and placed into 250 mL glass pots, which were filled with 3% NaCl and 0.12% ascorbic acid solution at 90 °C, sealed, commercially sterilized at 97 °C for 20 min and cooled to room temperature (Embrapa, 2000).

2.3. Mushroom in vitro digestion and potential bioaccessibility

Fresh, cooked and canned mushrooms were submitted to *in vitro* digestion according to the method described by Ariza et al. (2018) with a few modifications. Briefly, 5 g of mushroom were ground with 10 mL acidified distilled water (6 mol/L HCl, pH 1.8) for 30 s in an Ultra-turrax-T-25 (IKA, Staufen, Germany), followed by the addition of 1.0 mL pepsin solution (3.67 mg/mL, 8.030 units) and incubation at 37 °C for 120 min under continuous shaking (Cientec, Belo Horizonte, MG, Brazil). Pepsin activity was ended by increasing the pH to 7.8, using 3.3 mL of saturated solution of $NaHCO_3$. The extract was, then, purified by centrifugation at 5000*g* for 10 min (Excelsa Baby II 206-R, Fanen, São Paulo, Brazil) and stored at −80 °C until analysis (*in vitro* gastric digestion)

For the *in vitro* intestinal digestion, 5 g of mushroom were submitted to gastric digestion, as previously described. After raising pH to 7.8, using saturated solution of $NaHCO_3$, 1 mL solution containing pancreatin (45 mg/mL) and bile salt (281.2 mg/mL) was added and the mixture was incubated at 37 °C for 120 min under continuous shaking. Afterwards, pancreatin activity was ended by immersion in an ice bath. Samples were purified by centrifugation at 5000*g* for 10 min and the supernatant was collected and stored at −80 °C, until analysis. Blanks, without addition of sample, were used throughout the digestion processes to make sure there was no interference.

2.4. Methods of analysis

2.4.1. Moisture and crude protein contents

The moisture and crude protein contents of fresh, cooked and canned mushrooms were determined by oven-drying at 105 °C and micro-Kjeldahl, respectively (AOAC, 2012). Crude protein was calculated by multiplying total nitrogen content by 4.38 (Kalač, 2013).

2.4.2. Bioactive amines, amino acid and ammonia in mushrooms

Free bioactive amines, amino acids and ammonium ions were extracted from 2 g of fresh, cooked and canned mushrooms with 3 mL TCA (1.6%). The samples were agitated for 2 min in a shaker at 200 rpm and centrifuged at 7000*g* for 4 min at 4 °C. This step was

repeated twice. The supernatants were collected and filtered through Whatman #1 filter into a 25-mL volumetric flask (Reis, Guidi, Fernandes, Godoy, & Gloria, 2020). No extraction was needed for the fractions which resulted from the *in vitro* digestion.

Prior to pre-column derivatization of the extracts, the internal standard L-norvaline (50 mmol/L) was added to the mushroom extract (40 μL of internal standard + 9.960 mL of mushroom extract) and also to the *in vitro* digestion fractions (4 μL of internal standard + 996 μL of each *in vitro* digestion fractions). An aliquot of these extracts (500 μL) were neutralized using 300 μL NaOH (0.1 mol/L). After homogenization, 5 μL of the neutralized extracts were mixed with 30 μL of AccQ.Fluor® borate buffer and 15 μL AQC, it was allowed to rest for 1 min and then, it was heated in a water bath at 55 °C for 10 min. The extract was filtered using PTFE 0.22 μm pore size membrane (Minisart SRP 4®, Sartorius, Gottingen, Germany) and analyzed by UPLC (Reis et al., 2020).

The amines, amino acids and ammonia were separated according to a liquid chromatographic method (Reis et al., 2020). A Waters AcquityTM Ultra Performance LC (UPLC) system (Waters, Milford, MA, USA) equipped with an AcquityTM tunable ultraviolet (TUV) detector (249 nm) was used, with a CSH C18 column (50 × 2.1 mm, 1.7 μm id, Acquity UPLC). The solvent system consisted of A – 0.01 mol/L sodium acetate adjusted to pH 4.80 with acetic acid and B – acetonitrile at gradient elution. The injection volume was 2 μL and the gradient elution was operated at a flow rate of 0.9 mL/min as follows: initial–2.5 min/0–0% B; 2.8–4.5 min/0–3% B; 4.5–10.0 min/3–30% B; 10.0–11.0 min/30–100% B; 11.0–11.75 min/100–100% B; 11.75–12.5 min/100–0% B, and further re-equilibration at initial conditions for another 2.5 min, total cycle time of 15 min until the next injection. The concentration of bioactive amines, amino acids and ammonia was calculated by interpolation in the respective analytical curves ($R^2 \geq 0.996$) and the recovery of the internal standard was also used in the calculation. The results were expressed in mg/100 g of mushroom.

2.5. Statistical analysis

The results were submitted to Shapiro Wilk test for normality and Levene's test for homoscedasticity. Then, the data was submitted to analysis of variance and the means were compared by the Tukey test at 95% of probability. Two multivariate exploratory techniques – Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) – were applied for the characterization of the *in vitro* bioaccessibility of fresh and processed *A. bisporus* mushroom regarding free amino acids and bioactive amines (Granato, Santos, Escher, Ferreira, & Maggio, 2018). All data were analyzed using the Past 3.19 software (UIO, Oslo, Norway).

3. Results and discussion

3.1. Characterization of fresh mushroom

Fresh *Agaricus bisporus* mushrooms had high moisture content (92.9 g/100 g) and low amounts of protein (1.84 g/100 g), like literature values (Kalač, 2014) but lower protein compared to Solano-Aguilar et al. (2018) – 2.72 g/100 g. Among the 18 amino acids investigated, 14 were present in fresh *A. bisporus* (alanine, aspartic acid, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine), whereas arginine, cystine, methionine and tryptophan were not detected (Table 1). Among the free amino acids present, eight are essential amino acids (histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tyrosine and valine). This finding brings about the nutritional potential of this mushroom as a source of essential amino acids, which are required in the diet to prevent nitrogen imbalance, associated with several adverse health effects including weight loss, impaired growth, and other clinical symptoms (Marchini et al., 2016). The profile of amino acids found in *A. bisporus* is like literature data. However, arginine, methionine and tryptophan, which were not found in this study, were reported by some authors (Li et al., 2011; Pei et al., 2014; Rotola-Pukkila et al., 2019; Tsai, Tsai, & Mau, 2007).

The mean total level of free amino acids in *A. bisporus* mushroom was 398.8 mg/100 g. This result is within values reported in the literature (Li et al., 2011; Pei et al., 2014; Tsai et al., 2007); however, Rotola-Pukkila et al. (2019) reported higher levels (1006 mg/100 g). Alanine (93.8 mg/100 g) and glutamic acid (86.0 mg/100 g) were the

Table 1
Profile and level of free bioactive amines, amino acids and ammonia (mg/100 g) in fresh, cooked and canned mushrooms before and after *in vitro* gastric and gastric-intestinal digestions.

Class/Analyte	Mushroom (*A. bisporus*)			Gastric digestion			Gastric-intestinal digestion		
	Fresh	Cooked	Canned	Fresh	Cooked	Canned	Fresh	Cooked	Canned
Bioactive amines									
Spermidine	7.2 ± 0.2^{a}	7.1 ± 0.6^{a}	6.2 ± 0.1^{b}	7.7 ± 1.4^{a}	7.6 ± 0.1^{a}	6.5 ± 0.1^{b}	7.4 ± 0.8^{a}	7.6 ± 0.4^{a}	6.2 ± 0.1^{b}
Amino acids									
Aspartic acid	25.7 ± 0.8^{d}	14.9 ± 1.0^{e}	11.6 ± 0.2^{e}	44.8 ± 0.6^{b}	17.4 ± 0.1^{e}	17.2 ± 0.3^{e}	65.2 ± 6.2^{a}	35.7 ± 1.2^{c}	32.4 ± 2.6^{c}
Alanine	93.8 ± 2.1^{c}	59.8 ± 3.5^{d}	43.5 ± 0.6^{e}	148.8 ± 0.6^{b}	55.6 ± 0.3^{de}	59.7 ± 0.3^{d}	170.6 ± 19.9^{a}	83.4 ± 1.9^{c}	80.8 ± 6.9^{c}
Arginine	nd^{d}	nd^{d}	nd^{d}	23.9 ± 1.2^{c}	nd^{d}	nd^{d}	55.6 ± 4.4^{b}	81.6 ± 1.4^{a}	87.8 ± 6.0^{a}
Glutamic acid	86.0 ± 2.9^{cd}	51.5 ± 3.2^{e}	54.8 ± 1.1^{e}	138.9 ± 16.0^{b}	94.1 ± 2.5^{cd}	75.4 ± 1.4^{d}	185.2 ± 11.2^{a}	158.6 ± 4.4^{b}	105.6 ± 9.7^{c}
Glycine	10.8 ± 0.4^{c}	7.4 ± 0.4^{d}	6.3 ± 0.5^{d}	16.4 ± 0.1^{b}	7.2 ± 0.4^{d}	7.8 ± 0.1^{d}	26.7 ± 1.7^{a}	16.8 ± 0.3^{b}	16.7 ± 1.6^{b}
Histidine	9.4 ± 0.2^{d}	6.8 ± 0.6^{e}	6.0 ± 0.2^{e}	15.3 ± 0.5^{c}	7.2 ± 0.1^{de}	6.9 ± 0.1^{de}	27.6 ± 0.9^{a}	22.0 ± 0.6^{b}	21.4 ± 1.5^{b}
Isoleucine	17.3 ± 0.7^{d}	11.2 ± 0.6^{e}	9.1 ± 0.1^{e}	43.6 ± 1.2^{b}	12.2 ± 0.1^{de}	11.9 ± 0.1^{e}	62.5 ± 5.2^{a}	37.1 ± 0.7^{c}	36.3 ± 2.0^{c}
Leucine	29.4 ± 0.9^{d}	18.9 ± 1.1^{e}	15.7 ± 0.1^{e}	71.6 ± 1.4^{c}	20.9 ± 0.1^{de}	19.9 ± 0.1^{de}	97.0 ± 9.4^{a}	90.0 ± 1.3^{b}	93.0 ± 5.1^{b}
Lysine	19.0 ± 0.6^{c}	15.1 ± 0.6^{c}	12.5 ± 0.1^{c}	39.3 ± 0.1^{b}	16.7 ± 0.1^{c}	13.9 ± 0.1^{c}	70.3 ± 9.8^{a}	87.1 ± 1.5^{a}	85.8 ± 3.9^{a}
Methionine	nd^{c}	nd^{c}	nd^{c}	15.2 ± 0.1^{b}	nd^{c}	nd^{c}	20.8 ± 2.1^{a}	16.1 ± 0.3^{b}	20.9 ± 0.3^{a}
Phenylalanine	20.2 ± 1.0^{c}	12.9 ± 0.7^{d}	10.5 ± 0.1^{d}	47.1 ± 1.9^{b}	15.2 ± 0.1^{cd}	14.8 ± 0.4^{cd}	64.2 ± 6.7^{a}	69.5 ± 2.0^{a}	71.1 ± 4.8^{a}
Proline	19.8 ± 1.0^{c}	13.2 ± 0.8^{d}	13.5 ± 0.1^{d}	37.5 ± 0.9^{b}	13.4 ± 0.1^{d}	14.6 ± 0.1^{d}	43.1 ± 4.5^{a}	20.0 ± 1.0^{c}	19.4 ± 1.4^{c}
Serine	16.0 ± 0.9^{d}	15.6 ± 1.4^{d}	14.8 ± 2.7^{d}	24.4 ± 0.3^{c}	27.1 ± 0.8^{c}	21.6 ± 0.6^{c}	44.0 ± 2.9^{b}	49.4 ± 1.4^{a}	38.1 ± 3.7^{b}
Threonine	24.1 ± 0.8^{bc}	17.2 ± 1.0^{d}	14.1 ± 0.2^{d}	41.0 ± 2.4^{b}	18.5 ± 0.1^{cd}	16.6 ± 0.1^{d}	59.8 ± 5.6^{a}	36.8 ± 0.8^{b}	32.2 ± 2.3^{b}
Tyrosine	7.1 ± 0.3^{de}	11.2 ± 0.7^{cd}	9.8 ± 0.2^{cd}	4.4 ± 0.7^{e}	12.5 ± 0.1^{c}	10.9 ± 0.1^{cd}	23.2 ± 0.3^{b}	70.4 ± 1.5^{a}	73.1 ± 4.5^{a}
Valine	20.2 ± 0.8^{d}	13.2 ± 1.1^{e}	9.5 ± 0.4^{e}	52.2 ± 4.4^{b}	13.5 ± 0.3^{de}	11.7 ± 0.1^{e}	76.3 ± 5.6^{a}	44.0 ± 1.0^{c}	39.4 ± 2.5^{c}
Total	398.8 ± 13.2^{d}	268.8 ± 16.4^{e}	231.9 ± 7.7^{e}	740.5 ± 59.0^{c}	331.5 ± 2.8^{de}	302.9 ± 2.1^{de}	1170.1 ± 90.8^{a}	917.3 ± 14.4^{b}	853.9 ± 66.8^{bc}
Ammonia	2.7 ± 0.3^{e}	2.9 ± 0.3^{e}	2.7 ± 0.1^{e}	5.2 ± 0.8^{b}	3.2 ± 0.1^{de}	4.0 ± 0.3^{cd}	6.6 ± 0.8^{a}	4.5 ± 0.1^{bc}	4.5 ± 0.3^{bc}

Means were calculated using zero as nd (not detected). Means with different letters in the same line are significantly different (Tukey test, $p \leq 0.05$).

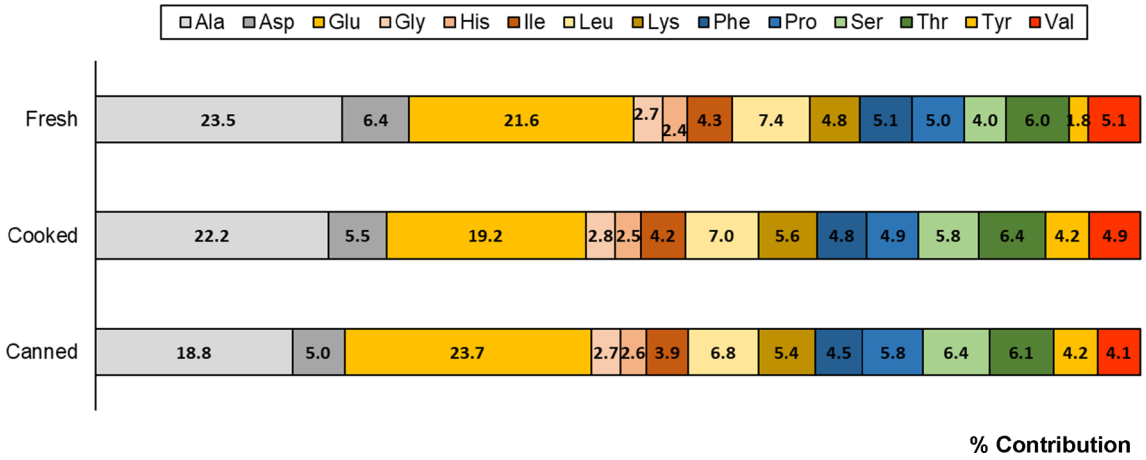

Fig. 1. Contribution (%) of each free amino acid to total levels in fresh, cooked and canned *Agaricus bisporus* mushrooms. Ala – alanine , Asp – aspartic acid, Glu – glutamic acid, Gly – glycine, His – histidine, Ile – isoleucine, Leu – leucine, Lys – lysine, Phe – phenylalanine, Pro – proline, Ser – serine, Thr – threonine, Tyr – tyrosine, Val – valine.

amino acids present at highest levels, representing 23.5% and 21.6% of total levels, respectively (Fig. 1). Aspartic acid, leucine, phenylalanine, proline, threonine and valine were the amino acids present at intermediate levels (20–30 mg/100 g), representing 5–7.5% of total levels, each. The other amino acids were present at levels below 19 mg/100 g, representing less than 5% of total levels. Similar results were reported in the literature (Li et al., 2011; Rotola-Pukkila et al., 2019); however, Tsai et al. (2007) and Pei et al. (2014) found histidine to be the predominant amino acid in *A. bisporus* mushroom. In fact, there are several factors that can affect the profile and levels of amino acids and other chemical components in mushroom, including conditions during production and processing and also the stages of maturity and of development of the fruiting bodies (Ming et al., 2014; Reis et al., 2020).

Besides being relevant from the nutritional point of view, some free amino acids can affect food flavor, especially with respect to sweetness, bitterness, and umami tastes (Li et al., 2011; Pei et al., 2014; Rotola-Pukkila et al., 2019; Tsai et al., 2007). According to Table 2, there is predominance of sweet (164.5 mg/100 g), followed by umami (111.7 mg/100 g) amino acids, characterized by the expressive levels of alanine and glutamic acid, respectively.

Among the ten bioactive amines investigated (spermine, spermidine, agmatine, putrescine, cadaverine, histamine, tryptamine, serotonin, tyramine and phenylethylamine), only spermidine was found in *A. bisporus*. The occurrence of spermidine in *A. bisporus*, as well as in other mushrooms and food matrices, has been reported in the literature (Dadáková et al., 2009; Kalač, 2013; Reis et al., 2020) and its presence is associated with the diverse and relevant roles on cellular metabolism and growth. The mean level of spermidine found in the mushroom was 7.2 mg/100 g, which is considered by Kalač (2014) a high source of polyamines (> 1 mg/100 g).

Several amino acids, which are precursors of bioactive amines, were available as free amino acids in the mushroom. For example, phenylalanine, lysine, histidine and tyrosine were present, and they are the precursors of phenylethylamine, cadaverine, histamine, and tyramine, which can be formed by the activity of the respective decarboxylases (EFSA (European Food Safety Authority), 2011; Gloria, 2006; Papageorgiou et al., 2018). Thus, *A. bisporus* would have the potential for the formation of these biogenic amines. Since amino acid decarboxylase activity is widespread in contaminating microorganisms, good manufacturing practices, including hygienic sanitary conditions during production and processing, are necessary for the quality and safety of mushroom.

Small amounts of ammonia were quantified in fresh mushrooms (2.7 mg/100 g). In fact, ammonia can be naturally present in fruiting bodies of *A. bisporus*, which is typical of Basidiomycetes species (Chang & Miles, 2004). Furthermore, ammonia can accumulate due to protein hydrolysis and, therefore, it can be an index of amino acid degradation (Kim et al., 2009).

Table 2
Levels (mg/100 g) of free amino acids in fresh, cooked and canned *A. bisporus* mushrooms according to taste characteristics.

Amino acids/taste	Levels (mg/100 g)		
	Fresh	Cooked	Canned
Umami	111.7 ± 2.6[a]	66.4 ± 3.0[b]	66.5 ± 0.6[b]
Sweet	164.5 ± 3.0[a]	113.2 ± 4.5[b]	92.3 ± 2.2[c]
Bitter	96.4 ± 2.9[a]	62.9 ± 3.0[b]	50.9 ± 0.7[c]
Tasteless	26.2 ± 0.8[a]	26.2 ± 1.1[a]	22.3 ± 0.3[b]

Means with different letters in the same line are significantly different (Tukey test, $p \leq 0.05$).
Taste amino acids: Umami – aspartic + glutamic acids; Sweet – alanine + glycine + proline + serine + threonine; Bitter – arginine + histidine + isoleucine + leucine + phenylalanine + valine; and Tasteless – lysine + tyrosine.

3.2. Influence of cooking and canning

The effect of cooking and canning on free amino acids and bioactive amines in *A. bisporus* was investigated for the first time. Cooking and canning did not affect the moisture content ($p > 0.05$) of the final products (92.85 ± 0.12 and 92.81 ± 0.06 g/100 g, respectively); therefore, the results for amino acids and amines were reported and compared on a wet weight basis. The protein content was also not affected by processing (~1.9 g/100 g).

After cooking and canning the mushroom, no changes were observed on the profile of free amino acids (Table 1), e.g. the same 14 amino acids were found. However, there was a significant decrease on total levels of amino acids, from 398.8 mg/100 g in fresh mushroom to 268.8 mg/100 g and 231.9 mg/100 g, which represents a reduction of 32.6% and 41.9% by cooking and canning, respectively. Similar results were observed during microwave, boiling and autoclave treatments of *A. bisporus* (Li et al., 2011). According to Rotola-Pukkila et al. (2019), the higher the cooking temperature, the higher are the losses of amino acids in *A. bisporus*, reaching 45% loss at 90 °C.

When comparing the levels of individual amino acids in fresh and processed samples (Table 1), most of them decreased during both heat treatments with lower levels observed in the canned compared to the cooked mushroom. The amino acids which were affected the most during cooking were aspartic acid (42% loss) and glutamic acid (40% loss); whereas aspartic acid (55% loss) and valine (53% loss) were the

most affected by canning. The least affected amino acids by cooking were lysine (21% loss) and histidine (28% loss); whereas during canning, the least affected were proline (32% loss) and lysine (34% loss). The amino acid losses observed in this study are smaller compared to those observed during *A. bisporus* cooking at 90 °C and mushroom soup preparation in an autoclave (Li et al., 2011; Rotola-Pukkila et al., 2019). However, even lower losses were observed during microwave cooking (Li et al., 2011).

Some amino acids decreased significantly during processing; however, the final levels were similar for cooked or canned mushrooms (glutamic acid, histidine, phenylalanine and proline). Other amino acids behaved in a different way, e.g. serine was not affected by either treatment; whereas the levels of tyrosine increased during processing, with higher levels observed for the cooked mushroom, compared to the canned product. The increase on tyrosine levels during thermal processing was also described by Li et al. (2011). The contribution of each amino acid to total levels in the cooked and canned products (Fig. 1) differed mainly for alanine (22.2 and 18.8%), glutamic acid (19.2 and 23.7%) and aspartic acid (5.5 and 5.0%, respectively).

The changes which occurred on the levels of free amino acids during processing can potentially affect the taste of the products (Table 2). The cooked and canned mushrooms had similar levels of umami amino acids; however, the canned mushroom had lower levels of the sweet taste amino acid – alanine. It also had lower levels of total amino acids with bitter taste (histidine + isoleucine + leucine + phenylalanine + valine). However, this hypothesis must be validated through sensory analysis.

Thermal processing is an effective way to preserve food, to improve shelf life, and to produce desirable physical or chemical changes and to improving digestibility, and palatability. However, the high temperatures used during cooking and canning can induce Maillard reaction. The loss of lysine (21 and 34%, for cooked and canned mushroom, respectively), which is the most reactive amino acid during non-enzymatic browning, suggests that this reaction may have happened, even though sulfite was added prior to canning (Huber & BeMiller, 2017). Furthermore, there can be loss of free amino acids by leaching, which can be enhanced at the lower pH values of the canned mushroom (pH $<$ 4.5).

The profile of bioactive amines was not affected by processing, as only spermidine was detected after cooking and canning. During cooking, the levels of spermidine were not affected; however, during canning, there was a 14% loss. Loss of spermidine through lixiviation in cooking water was described in the literature (Muñoz-Esparza et al., 2019). Despite this loss, canned mushrooms can still be considered a high source of spermidine (Kalač, 2014). This is important because dietary polyamines are essential for the maintenance of normal growth, maturation of the intestinal tract (Ali, Poortvliet, Strömberg, & Yngve, 2011) and are required during the periods of wound healing, post-operational recovery, liver regeneration, or compensatory growth of the lung or the gut (Kalač, 2014). No changes were observed for ammonia during thermal processing.

3.3. In vitro digestion of bioactive amines from A. bisporus

Among the ten bioactive amines analyzed, only spermidine was found in *A. bisporus* mushrooms after *in vitro* digestion (Table 1). Based on these results, there were no other bioactive amines in *A. bisporus* in the bound form that could have been released after *in vitro* digestion. Similar behavior was also observed for cooked and canned mushrooms. The content of spermidine remained unchanged throughout the *in vitro* gastric-intestinal digestions; what indicates that this polyamine was fully bioaccessible. Again, this same behavior was observed for both cooked and canned mushrooms, and, therefore, processing did not affect *in vitro* digestion of spermidine in fresh and processed *A. bisporus*.

The high bioaccessibility of spermidine from mushroom, either fresh or processed is important. This is so because of the contribution of this polyamine to health, including its role in the development of the immune system, wound healing, anti-inflammatory, antioxidant activity and cardio-protective effects (De Cabo & Navas, 2016; Handa, Fatima, & Mattoo, 2018; Kalač, 2014; Ramani, De Bandt, & Cynober, 2014; Sharma, Kumar, & Deshmukh, 2018).

3.4. In vitro digestion of amino acids from A. bisporus

3.4.1. Fresh mushroom

The amino acids profile observed for the fresh mushrooms after *in vitro* digestions of *A. bisporus*, both gastric and gastric-intestinal phases, are indicated in Table 1. After the *in vitro* digestion of fresh mushroom, two additional amino acids were detected besides the 14 that were present in fresh mushroom – arginine and methionine. Therefore, during *in vitro* digestion of fresh mushroom, additional amino acids were released.

When considering the total levels of free amino acids in fresh mushroom, after *in vitro* digestions compared to initial values (398.8 mg/100 g), significant increases (85.7 and 193.4%) were observed after gastric and gastric-intestinal digestions, reaching levels of 740.5 mg/100 g (40% of protein weight) and 1124.5 mg/100 g (61% of protein weight), respectively. These results indicate that during *in vitro* gastric and gastric-intestinal digestion, there is a significant release of amino acids. Although mushrooms have low protein content, there is a significant release of amino acids from the protein during digestion.

In vitro digestion released amino acids from the mushroom. *In vitro* gastric digestion of fresh mushroom resulted in 1.9-fold increase in total amino acids, 2-fold increases of isoleucine, leucine, phenylalanine, valine and proline; and 1.5-fold increases of aspartic acid, alanine, histidine, serine and threonine; arginine and methionine were released and there was 40% loss of tyrosine.

After *in vitro* gastric digestion of fresh mushroom, alanine (148.8 mg/100 g) and glutamic acid (138.9 mg/100 g) were the major amino acids, accounting for 20.1% and 18.8%, respectively, of the total content. This result changes after the complete *in vitro* digestion (gastric-intestinal) and glutamic acid (185.2 mg/100 g) is the most released amino acid followed by alanine (170.6 mg/100 g), which represented 15.8% and 14.6% of the total amount of amino acids released.

Regarding the essential amino acids released from *A. bisporus* after digestion in the gastric phase, the essential amino acid content ranged from 4.4 (tyrosine) to 71.6 mg/100 g (leucine), which represented 0.6–9.7% for each essential amino acid of the total amino acid content released at this stage of digestion. When compared to the free amino acid content in the mushrooms, digestion in the gastric phase provided an increase in the release of most of the essential amino acids, except for tyrosine that had a decrease in its content. This decrease in tyrosine's content may be related to the sensitivity of this amino acid to the acidic conditions in the gastric phase (Pickering & Newton, 1990). At the end of the digestion, the essential amino acid content ranged from 23.2 (tyrosine) to 97.0 mg/100 g (leucine), which represented a variation of 2.0–8.3% for these amino acids, respectively, compared to the initial contents.

The profile of amino acids remained unchanged after gastric digestion. This result can be explained by the delayed digestion of protein due to the presence, even at low levels, of negatively charged polysaccharides (Hu et al., 2017), and take into consideration that mushrooms have several different types of low digestible and non-digestible carbohydrates, including β-glucans, raffinose, oligosaccharides, and resistant starch (Hess, Wang, Gould, & Slavin, 2018). In addition, this research did not perform the *in vitro* oral digestion, which could have contributed to the initial digestion of starch. However, at the end of the complete *in vitro* digestion (gastric-intestinal), arginine and methionine were detected. It is possible that these two amino acids were released by pancreatin activity in the intestinal phase. Another possibility would be the release of an amino acid that is already free but trapped into the matrix. It is possible that digestion disrupted the food matrix thereby

releasing the amino acid (Tavano, 2013). The profile of spermidine was not altered after *in vitro* digestion. The release of histidine, tyrosine, phenylalanine and lysine in fresh mushroom by the *in vitro* digestion process may contribute to the formation of bioactive amines like histamine, tyramine, phenylethylamine and cadaverine by the decarboxylation of these amino acids through the gut microbiota (Diether & Willing, 2019).

3.4.2. Cooked and canned mushrooms

The total levels of free amino acids after *in vitro* digestions of cooked and canned mushrooms increased slightly after gastric digestion (23.3 and 30.6%, respectively), but it increased substantially after complete digestion (241.3 and 268.2%, respectively). These changes were higher than observed in fresh mushroom (Table 1). These results suggest that processing facilitated the release of amino acids after *in vitro* digestion.

After *in vitro* gastric digestion of the cooked and canned mushroom there was a mean 1.2-fold increase of total amino acids. Higher increases were observed for aspartic acid, alanine, glutamic acid and serine (~1.3-fold) and no difference was observed for the other amino acids. No difference was observed between cooked or canned mushrooms ($p > 0.05$). Glutamic acid (94.1 mg/100 g for cooked and 75.4 mg/100 g for canned) and alanine (55.6 mg/100 g for cooked and 59.7 mg/ 100 g for canned) were the amino acids which contributed the most to total amino acid content. Glutamic acid contributed with 28.4% and 24.9% of the total amount of amino acids released in the cooked and canned mushroom, respectively. And alanine contributed with 16.8 and 19.7% of the total content of amino acids released in the cooked and canned mushroom, respectively.

The total levels of amino acids after *in vitro* gastric-intestinal digestions increased 3.3-fold for the fresh and processed mushroom. Lower increases were observed for alanine and proline (~1.6-fold). Aspartic acid, glutamic acid, glycine, serine and threonine increased ~2.5-fold; and histidine, isoleucine and valine increased ~3.5-fold. Leucine, lysine and tyrosine had different increasing rates for fresh (~3.5-fold), cooked (~5.6-fold) and canned (~6.8-fold) mushroom. In addition, methionine and arginine, which were not detected prior to *in vitro* digestion, showed up. The first was present at similar amounts in all final products (~19 mg/100 g), whereas arginine was present at increasing levels for *in vitro* gastric-intestinal digested fresh, cooked and canned mushroom (55.6, 81.6 and 87.8 mg/100 g, respectively).

Glutamic acid was the amino acid at the highest content detected after the complete *in vitro* digestion of processed mushrooms (158.6 mg/100 g for cooked and 105.6 mg/100 g for canned), which represented a contribution of 17.2% and 12.4% to the total amino acid contents, respectively. Other amino acids including arginine, leucine, lysine and alanine also presented high contents, ranging from 81.6 mg/100 g (arginine) to 90 mg/100 g (leucine) released in the cooked mushroom and 80.8 mg/100 g (alanine) to 93.0 mg/100 g (leucine) released in the canned mushroom.

Regarding the essential amino acid release after *in vitro* digestion, leucine and lysine were the amino acids that contributed the most to the total free amino acid content. Tyrosine and phenylalanine were also detected, representing a contribution of 7.6% (phenylalanine) and 7.7% (tyrosine) in cooked and 8.3% (phenylalanine) and 8.6% (tyrosine) in canned mushroom to the total of amino acids released after *in vitro* digestion. The other essential and non-essential amino acids quantified, contributed each, with less than 5% of the total of amino acids released after *in vitro* digestion.

Processing is critical to increase mushroom shelf life. Even though there was a decrease on the levels of free amino acids by thermal processing (cooking or canning), at the end of the *in vitro* digestion there was significant release of amino acids (Table 1). Thus, thermal processing, in addition to increasing shelf life of mushroom, did not affect to a great extent the contents of amino acid released after digestion, thereby maintaining the nutritional value.

The polyamine spermidine was not affected by *in vitro* digestion of the fresh, cooked and canned mushrooms. This polyamine was released by digestion in the gastric phase and there was no change after intestinal digestion. At the end of the *in vitro* digestion, 7.6 and 6.2 mg/100 g spermidine were released in cooked and canned mushrooms, respectively. When compared to the content in fresh mushrooms at the end of the *in vitro* digestion, the content of this polyamine remained unchanged, so spermidine was also fully bioaccessible in processed mushrooms.

With respect to ammonia, there was a significant increase during digestion of fresh and cooked mushrooms (Table 1), this may reflect the influence of digestive enzymes on protein hydrolysis and, consequently, ammonia release.

The comparison between the free amino acids released after *in vitro* digestion per gram of mushroom protein to the amino acid requirements of adults in mg per g protein (FAO/WHO, 2013) is presented in Table 3. The values obtained after mushroom digestion are adequate for most of the required amino acids, which emphasizes the value of the protein from this mushroom. For the fresh sample, isoleucine, tyrosine + phenylalanine and threonine are above, while histidine and valine are close to the recommendations. Leucine, lysine and methionine contribute with more than 50% of each respective amino acid recommendation (FAO/WHO, 2013). Therefore, *A. bisporus* has good quality proteins, despite the high moisture and low protein content.

Table 3
Levels of essential amino acids in *Agaricus bisporus* mushrooms (fresh, cooked and canned) after *in vitro* gastric-intestinal digestion in relation to maintenance of amino acid pattern (mg/g protein) for adults (FAO/WHO, 2013).

Essential Amino acids	Amino acid level released (mg) after *in vitro* digestion per g of mushrooms protein			FAO amino acid pattern for adults (mg/g protein)*
	Fresh	Cooked	Canned	
Histidine	14	11	11	15
Isoleucine	32	19	18	30
Leucine	49	45	47	59
Lysine	35	44	43	45
Methionine + Cysteine (SAA)	11	8	11	22
Phenylalanine + Tyrosine (AAA)	44	70	72	38
Threonine	30	19	16	23
Tryptophan	nd	nd	nd	6
Valine	38	22	20	39

SAA – sulphur amino acids; AAA – aromatic amino acids; nd – not detected.

3.5. Multivariate analyses

Multivariate analyses were applied to estimate the influence of thermal processing and *in vitro* digestion on the contents of bioactive amines, amino acids and ammonia in fresh, cooked and canned *A. bisporus* mushroom (Fig. 2). Before PCA model was built, data were auto scaled, and no other preprocessing was used. The principal component (PC) model was built and two principal components (PC1 and PC2), explained 97.3% of the variance. The first PC explained 78.9% of the variance (Fig. 2a), and it differentiated cooked and canned mushroom submitted to gastric-intestinal digestion – GIC and GIP, respectively, and fresh samples after gastric – GF and gastric-intestinal – GIF phases (positive PC1 values) from the other treatments. The differences were mainly due to amino acids, including glutamic acid, leucine, alanine, arginine, and lysine, which were higher in these compounds (Fig. 2b). Based on these results, spermidine and the ammonia had little discrimination power.

PC2 explained 18.4% of the variance, and it differentiated the cooked and canned gastric-intestinal samples (GIP and GIC) from fresh gastric and fresh gastric-intestinal samples (GF and GIF), and also cooked, canned, cooked gastric and canned gastric samples (C, P, CG, PG) from fresh samples (F). The first groups had higher levels of

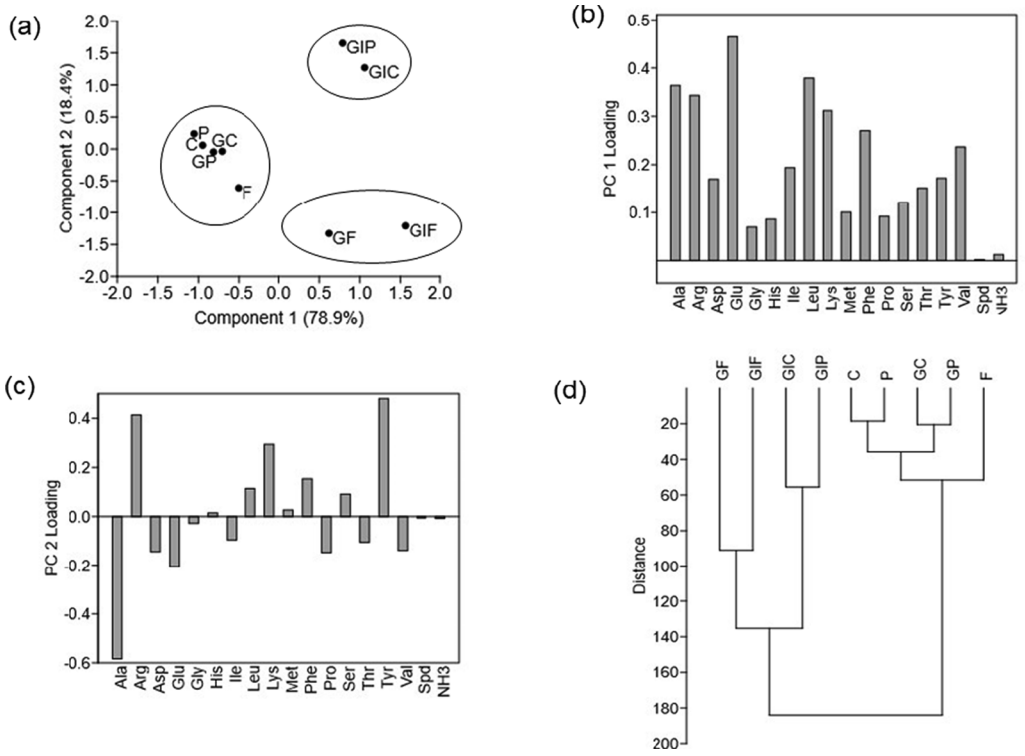

Fig. 2. Scatter plot (a), PC1 loading plot (b), PC2 loading plot (c) and dendrogram (d) obtained by Principal Component Analyses (PCA) and Hierarquical Cluster Analyses (HCA) of the mean levels of free bioactive amines, amino acids and ammonia in fresh (F), cooked (C) and canned (P) *Agaricus bisporus* mushroom before and after *in vitro* gastric and gastric-intestinal digestions. GF– fresh mushroom after gastric phase, GC – cooked mushroom after gastric phase, GP – canned mushroom after gastric phase, GIF – fresh mushroom after gastric and intestinal phases, GIC – cooked mushroom after gastric and intestinal phases, GIP – canned mushroom after gastric and intestinal phases. Ala – alanine, Arg – arginine, Asp – aspartic acid, Glu – glutamic acid, Gly – glycine, His – histidine, Ile – isoleucine, Leu – leucine, Lys – lysine, Met – methionine, Phe – phenylalanine, Pro – proline, Ser – serine, Thr – threonine, Tyr – tyrosine, Val – valine, Spd – spermidine, and NH_3 – ammonia.

tyrosine, arginine and lysine and lower levels of alanine and glutamic acid compared to the second ones (Fig. 2c).

Hierarchical Cluster Analysis (HCA) using algorithm paired group was built and presented 92.8% of cophenetic correlation coefficient. It confirmed PCA results, separating the samples into three main groups (Fig. 2d). The first group comprised of cooked and canned mushrooms submitted to gastric-intestinal digestion (GIC and GIP), which were characterized by the highest levels of arginine, lysine, phenylalanine and tyrosine. The second cluster was formed by the fresh samples which underwent gastric (GF) and gastric-intestinal (GIF) digestions, and had the highest levels of glutamic acid and alanine and high levels of arginine and tyrosine. The third cluster contained the remaining samples, which were characterized by absence of arginine and methionine and lower levels of all the other amino acids.

4. Conclusions

Fresh *Agaricus bisporus* was characterized by the presence of 14 free amino acids, with prevalence of alanine and glutamic acid, only one amine – spermidine and low levels of ammonia. Processing, which improves mushroom shelf-life, caused a decrease on total levels and most individual amino acids, except for lysine, serine and tyrosine, which were not affected by heat treatment. No significant difference between cooked and canned mushroom was observed for most amino acids, except for alanine that had 36% and 54% losses after cooking and canning, respectively. Spermidine contents were maintained during cooking, but it decreased during canning. The absence of other bioactive amines in this mushroom can be the object of new studies in order to develop foods and formulations with highest spermidine content for functional properties.

In vitro digestion released amino acids from the mushroom. *In vitro* gastric digestion of fresh mushroom resulted in ~2-fold increase in total amino acids, isoleucine, leucine, phenylalanine, valine and proline; and 1.5-fold increases of aspartic acid, alanine, histidine, serine and threonine; arginine and methionine were released and there was 40% loss of tyrosine. For cooked and canned mushroom *in vitro* gastric digestion caused a mean 1.1-fold increase in amino acids, and no difference was observed for cooked or canned mushrooms.

In vitro intestinal digestion of fresh and cooked mushroom provided additional significant release of amino acids. The final product from the *in vitro* gastric-intestinal digestion from fresh mushroom had 2.9-fold increase in total amino acids, and higher increases of isoleucine, leucine, phenylalanine and valine (2.4-fold). Processed mushroom had higher increase in total amino acids (3.5-fold) compared to fresh. There were high increases on leucine (4.8- and 5.9-fold), lysine (5.8- and 6.8-fold) and tyrosine (6.2- and 7.5-fold) for cooked and canned mushroom, respectively. It also released argine and methionine, which were not present in fresh mushroom.

Spermidine in *A. bisporus* was fully bioaccessible, a novelty for amines and mushroom. The amino acid levels obtained after mushroom digestion are adequate for most of the required amino acids, which emphasizes the value of the protein from this mushroom.

Multivariate analysis separated processed and *in vitro* digested mushroom into three clusters based on amino acids, emphasizing the higher abundance of glutamic acid, leucine, lysine, arginine and tyrosine after *in vitro* gastric-intestinal digestion.

CRediT authorship contribution statement

Guilherme C.L. Reis: Conceptualization, Methodology, Investigation, Writing - original draft. **Bruno M. Dala-Paula:** Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **Olga L. Tavano:** Methodology, Writing - review & editing. **Leticia R. Guidi:** Methodology, Validation, Investigation, Writing - review & editing. **Helena T. Godoy:** Methodology, Investigation. **Maria Beatriz A. Gloria:** Conceptualization, Methodology, Investigation, Writing - review & editing, Funding acquisition, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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