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Utilização de substrato residual de cogumelos (SRC) em novos ciclos de produção e avaliação da atividade antioxidante de *Lentinus crinitus* e *Pleurotus* spp.

Use of spent mushroom substrate (SMS) in new production cycles and evaluation of the antioxidant activity of *Lentinus crinitus* and *Pleurotus* spp.

Utilización de sustrato residual de hongos (SRH) agotado en nuevos ciclos de producción y evaluación de la actividad antioxidante de *Lentinus crinitus* y *Pleurotus* spp.

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Resumo

Lentinus crinitus e *Pleurotus* spp. são cogumelos que podem ser produzidos em subprodutos agroindustriais lignocelulósicos. Após a produção dos cogumelos, o substrato contendo o micélio constitui o substrato residual de cogumelos (SRC). Sua reutilização em novos ciclos de produção gera perspectivas para uma cadeia produtiva mais eficiente. O SRC de *L. crinitus* foi utilizado para produzir *Pleurotus* spp. e o processo inverso foi realizado, para verificar a possibilidade de reutilização do SRC. A atividade antioxidante do SRC gerado nos dois ciclos de cultivo e dos cogumelos colhidos foi avaliada. O cultivo em SRC diminuiu a produtividade, a eficiência biológica e o consumo de substrato. *P. djamor* e *P. ostreatus* produzidos em SRC de *L. crinitus* apresentaram redução na produtividade de 4,4 e 4,2 vezes, respectivamente. A eficiência biológica seguiu a mesma tendência de queda. A sucessão microbiana reduziu a atividade antioxidante do SRC, conforme avaliado pelos métodos FRAP (poder antioxidante de redução férrica) e BCLA (sistema de co-oxidação de beta-caroteno/ácido linoleico). Apenas a atividade antioxidante, avaliada pelo método de sequestro do radical DPPH (2,2-difenil-1-picrilhidrazil), do substrato de cultivo secundário (SRC) aumentou após o segundo ciclo de cultivo. Os cogumelos produzidos em cultivo secundário apresentaram maior atividade antioxidante pelo método DPPH do que os produzidos em cultivo primário. *L. crinitus* cultivado sucessivamente a *P. djamor* apresentou uma redução de 2,5 vezes na concentração inibitória (CI_{50}) em comparação com os cogumelos do cultivo primário. O crescimento em SRC também aumentou a atividade antioxidante dos cogumelos pelo método FRAP. *L. crinitus* cultivado em SRC de *P. djamor* e em SRC de *P. ostreatus* apresentou as maiores atividades antioxidantes pelo método FRAP, sete e 6,3 vezes superiores ao controle de referência, respectivamente. Apenas a atividade antioxidante pelo método BCLA dos cogumelos *P. ostreatus* cultivados em SRC de *L. crinitus* foi significativamente maior do que a dos cogumelos do cultivo primário. Apesar da redução na produtividade, o uso de SRC em uma nova composição de substrato aumentou a atividade antioxidante dos cogumelos. O SRC induziu o aumento da atividade antioxidante.
Palavras-chave: Antioxidante; basidiomiceto; sucessão microbiana; valorização de resíduos.

Abstract

Lentinus crinitus and *Pleurotus* spp. are mushrooms that can be produced in lignocellulosic agro-industrial by-products. After mushroom production, the substrate containing the mycelium constitutes the spent mushroom substrate (SMS). Its reuse in new production cycles generates prospects for a more efficient production chain. The SMS from *L. crinitus* was used to produce *Pleurotus* spp. and the reverse path was taken, to verify the possibility of SMS reuse. The antioxidant activity of the SMS generated in the two cycles of cultivation and the mushrooms harvested was evaluated. Cultivation in SMS decreased productivity, biological efficiency, and substrate consumption. *P. djamor* and *P. ostreatus* produced in SMS from *L. crinitus* showed a reduction in productivity of 4.4 and 4.2 times, respectively. Biological efficiency followed the same decreasing trend. The microbial succession reduced the SMS antioxidant activity by the FRAP (ferric reducing antioxidant power) and BCLA (beta-carotene/linoleic acid co-oxidation system) methods. Only the antioxidant activity by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method of SMS increased after the second cultivation cycle. Mushrooms produced in secondary cultivation have higher DPPH antioxidant activity than in primary cultivation. *L. crinitus* produced in succession to *P. djamor* presented a 2.5-

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fold reduction in inhibitory concentration (IC_{50}) compared to mushrooms from the primary cultivation. The growth in SMS also increased mushroom FRAP activity. *L. crinitus* grown in SMS of *P. djamor*, and SMS of *P. ostreatus* showed the highest antioxidant activities by FRAP, seven times and 6.3 times superior to reference control. Only the antioxidant activity by BCLA of *P. ostreatus* mushrooms grown in SMS of *L. crinitus* was significantly higher than the mushrooms from primary cultivation. Despite the reduction in productivity, the use of SMS in a new substrate composition increased the antioxidant activity of mushrooms. The SMS induced an increase in antioxidant activity.
Keywords: Antioxidant; basidiomycete; microbial succession; waste valorization.

Resumen

Lentinus crinitus y *Pleurotus* spp. son hongos que pueden producirse a partir de subproductos agroindustriales lignocelulósicos. Tras la producción de hongos, el sustrato que contiene el micelio constituye el sustrato residual de hongos (SRH). Su reutilización en nuevos ciclos de producción genera perspectivas para una cadena de producción más eficiente. El SRH de *L. crinitus* se utilizó para producir *Pleurotus* spp., y se llevó a cabo el proceso inverso para verificar la posibilidad de reutilizar el SRH. Se evaluó la actividad antioxidante del SRH generado en los dos ciclos de cultivo y de los hongos cosechados. El cultivo en SRH disminuyó la productividad, la eficiencia biológica y el consumo de sustrato. *P. djamor* y *P. ostreatus* producidos en SRH de *L. crinitus* mostraron una reducción en la productividad de 4,4 y 4,2 veces, respectivamente. La eficiencia biológica siguió la misma tendencia descendente. La sucesión microbiana redujo la actividad antioxidante del sustrato de cultivo secundario (SRH), según lo evaluado por los métodos FRAP (poder antioxidante reductor férrico) y BCLA (sistema de cooxidación de betacaroteno/ácido linoleico). Solo la actividad antioxidante, evaluada por el método de eliminación de radicales DPPH (2,2-difenil-1-picrilhidrazilo), del sustrato de cultivo secundario (SRH) aumentó después del segundo ciclo de cultivo. Los hongos producidos en cultivo secundario mostraron mayor actividad antioxidante por el método DPPH que los producidos en cultivo primario. *L. crinitus* cultivado sucesivamente a *P. djamor* mostró una reducción de 2,5 veces en la concentración inhibitoria (CI_{50}) en comparación con los hongos del cultivo primario. El crecimiento en SRH también aumentó la actividad antioxidante de los hongos por el método FRAP. *L. crinitus* cultivado en SRH de *P. djamor* y en SRH de *P. ostreatus* mostró las actividades antioxidantes más altas por el método FRAP, siete y 6,3 veces más altas que el control de referencia, respectivamente. Solo la actividad antioxidante de los hongos *P. ostreatus* cultivados en SRH de *L. crinitus* fue significativamente mayor, según lo medido por el método BCLA, que la de los hongos de cultivo primario. A pesar de la reducción en la productividad, el uso de SRH en una nueva composición de sustrato aumentó la actividad antioxidante de los hongos. El SRH indujo un aumento en la actividad antioxidante.
Palabras clave: Antioxidante; basidiomiceto; sucesión microbiana; valorización de residuos.

1. Introduction

World production of cultivated edible mushrooms has increased nearly 40-fold since the 1970s. In 2018 China alone produced 37.8 million tonnes of mushrooms and was the world's largest producer (Cefa, 2021), followed by the United States of America and the Netherlands (Kumla *et al.*, 2020). The most common edible mushrooms found worldwide are *Pleurotus ostreatus, Lentinula edodes, Agaricus bisporus, Flammulina velutipes*, and *Auricularia auricula* (Ma *et al.*, 2018). Among the various genera consumed, the five main genera originate

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about 85% of the world's mushroom supply. *Lentinula* is the main genus, contributing about 22% of the mushrooms cultivated worldwide and followed by *Pleurotus* spp., with six cultivated species constituting about 19% of world production, while *Auricularia* contributes about 17%. The other two genera, *Agaricus* and *Flammulina* account for 15 and 11% of the volume, respectively (Royse *et al.*, 2017).

Lentinus crinitus (L.) Fr. (Polyporaceae) is an edible mushroom native to Brazil with pantropical distribution (Silva Neto *et al.*, 2020). It is a saprophytic fungus that grows on decaying tree trunks (Pegler, 1977; Machado *et al.*, 2016) and produces mushrooms with nutritional and medicinal properties (Bertéli *et al.*, 2021a, 2021b). In addition, it has biotechnological applications such as lithium bioaccumulation as an alternative for making bio-enriched functional foods (Faria *et al.*, 2019) and as a dye decolorant (Tavares *et al.*, 2020). *L. crinitus* have been consumed by Amazonian ethnic groups such as the Yanomami (Sanuma-Roraima) and Txicão in Brazil, Uitoto, Muinane and Andoke (Caquetá) in Colombia, the rural population of Loreto in Peru and Hotï in the legal Amazon of Venezuela (Vargas-Isla *et al.*, 2013).

Pleurotus (Fr.) P. Kumm (Pleurotaceae) are saprophytic and comprise a wide variety of species, around 50, almost all of them edible. However, only a few are currently domesticated with the possibility of industrial production. It is a very versatile and adaptable genus known as Hiratake and smaller mushrooms as Shimeji (Bononi *et al.*, 1995). Fungi of the *Pleurotus* genus are known as oyster mushrooms. Examples include *Pleurotus florida, Pleurotus pulmonarius, Pleurotus eryngii*, and *Pleurotus ostreatus*. *Pleurotus* spp. are appreciated worldwide for their aroma and flavor and are also known for their nutritional properties (Rampinelli *et al.*, 2010; Fernandes *et al.*, 2015), being an important mushroom commercially (Maity *et al.*, 2011; Wang *et al.*, 2015). *Pleurotus* mushrooms, in general, are found in tropical and subtropical forests around the world and can be cultivated in a wide variety of substrates that contain cellulose, hemicellulose, and lignin due to the production of an enzyme complex composed of cellulases, hemicellulases, ligninases, and proteases, among other enzymes (Fernandes *et al.*, 2014). Thus,

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cultivating these mushrooms can represent an alternative of using agricultural by-products, preventing their accumulation in nature, and adding value, as mushrooms are foods of high economic value.

Lentinus and *Pleurotus*, due to their enzymatic complex, can grow on different substrates containing agro-industrial by-products such as coffee, rice and soybean husks, wheat bran, sugarcane bagasse, straw, and sawdust (Castro *et al.*, 2007). On the other hand, they produce bioactive compounds with antioxidant activity (Correa *et al.*, 2015; Bertéli *et al.*, 2021b). In addition, the metabolites from basidiomycetes as phenolic compounds, tocopherols, polysaccharides, and organic acids are present in the fruiting body and throughout the vegetative mycelium that constitutes the mycelial mass and permeates the cultivation substrate (Ito *et al.*, 1997; Mizuno *et al.*, 1999).

After a mushroom production cycle, the substrates are known as spent mushroom substrates (SMS). Despite research on their reuse, the material is usually discarded or used as organic fertilizer (Rinker, 2002; Andrews *et al.*, 2021). Sugarcane bagasse is one of the main substrates for growing mushrooms. It is a by-product of the sugar and alcohol industry used in the production of biofuels and electricity (Conab, 2021a). The 2019/2020 sugarcane harvest in Brazil was 634.01 million tons, generating 190.2 million tons of sugarcane bagasse (Conab, 2021a). This lignocellulosic material has been studied to produce several mushrooms such as *Pleurotus citrinopileatus, P. eryngii, P. florida*, and *P. ostreatus* (Pandey *et al.*, 2000). Soybean meal is a by-product derived from soybean oil extraction. It is rich in protein (~45%) and minerals (~20%) (Rieger *et al.*, 2008) with applications in soil fertilization, biodiesel production, animal feed, and mushroom cultivation (Eira and Minhoni, 1997). The Brazilian soybean crop 2020/2021 was 136 million tons, and 46.75 million tons of the grain were crushed, generating approximately 37.4 million tons of soybean meal (Conab, 2021b).

The use of SMS in new cultivation cycles is economically and environmentally viable, representing a way to associate the concept of circular economy in the production of edible mushrooms (Buendía *et al.*, 2017; Zied *et al.*, 2020; Andrews *et al.*, 2021). Furthermore, the reuse of SMS within the same

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process is attractive, as it allows optimization of the biomass and, therefore, tends to improve energy efficiency making possible a sustainable process (Zied *et al.*, 2020; Andrews *et al.*, 2021). However, maintaining the quality and standard of mushrooms and ensuring the viability of the production process are challenges that must be considered (Zied *et al.*, 2020). Furthermore, the use of SMS in the preparation of substrates for new cultivation cycles should consider aspects such as the need for enrichment and combination with other materials, the necessary substrates treatments, and the results (Zied *et al.*, 2020; Andrews *et al.*, 2021). However, studies on the reuse of SMS in new mushroom production create opportunities for producers to reuse this abundant by-product, in addition to helping in the prospecting of bioactive metabolites.

In this context, we first cultivated *L. crinitus, P. ostreatus*, and *P. djamor* and used the reformulated SMS for a new production cycle of these mushrooms. Therefore, this study aimed to reuse the SMS from the cultivation of *L. crinitus* to produce *Pleurotus* spp. and the reverse path was taken, to evaluate the antioxidant activity of mushrooms and SMS generated in the two stages of cultivation.

2. Material and Methods

2.1 Fungi and inoculum production

The strains used in this study were *Lentinus crinitus* (L.) Fr. U9-1; *Pleurotus ostreatus* (Jacq.) P. Kumm. U16-22 and *Pleurotus djamor* (Rumph. ex Fr.) Boedijn (U16-28).

The fungi were thawed and transferred to malt extract (20 g L^{-1}; Himedia®) and agar (5 g L^{-1}; Himedia®) culture medium, previously autoclaved (121 °C for 20 min) for recovery of the growth vigor (Tanaka *et al.*, 2019). The fungi were transferred and maintained in malt extract agar (MEA; 10 g L^{-1} agar and 20 g L^{-1} malt extract; Himedia®), previously autoclaved (121 °C for 20 min), at 28 ± 1 °C, in the dark, for seven days. MEA cylinders containing mycelium with homogeneous growth and without sectoring were used for inoculum production in wheat grain substrate.

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Wheat grains were cooked in excess volume of ultrapure water containing 1.5% calcium carbonate (mass/mass) to adjust the pH to 6.0. After water removal, the grains were transferred to polypropylene bags (30 cm wide x 40 cm high x 20 cm deep) with a bacterial filter (Micropore®; 5.5 cm^2) and then autoclaved at 121 °C for 2 h). MEA discs containing *L. crinitus, P. ostreatus*, or *P. djamor* mycelium were used to inoculate the wheat grains. The polypropylene bags were incubated at 28 ± 1 °C, in the dark until complete colonization (approximately ten days). Colonized wheat grains (2% of the total weight of the substrate) were used as an inoculum vehicle for the cultivation substrate.

2.2 Physicochemical analysis of agro-industrial by-products

Sugarcane bagasse (SB) and soybean meal (SM) were used in the substrate formulation. The Kjeldahl method was used to determine nitrogen concentration, ashes by burning at 550 ^oC, and moisture by drying at 105 ^oC until constant mass (Zenebon *et al.*, 2008). The carbon/nitrogen ratio (C/N) was calculated considering 50% of the dry organic mass as carbon mass (Gerrits, 1988; Mantovani *et al.*, 2007) and from the physicochemical analyses.

2.3 Primary and secondary cultivation substrates

The primary cultivation substrate (2 kg) was composed of sugarcane bagasse (SB) and soybean meal (SM) (19:1 and C/N = 46.36). The substrate was placed in polypropylene bags (30 cm wide x 40 cm high x 20 cm deep) with a bacterial filter (Micropore®, 5.5 cm^2), 80% humidity, pH 6, and autoclaved to 121 °C for 2 h. After autoclaving and cooling at room temperature, the culture bags were inoculated in a laminar flow chamber with 2% of wheat grains colonized with mycelium (mass/mass), heat-sealed, and kept at 28 ± 1 °C, in the dark, in growing room with a relative humidity of 80-90% until complete colonization of the substrate (30 days). When the mushroom harvest from the primary cultivation was completed, the spent substrate was prepared to be used in a new cultivation substrate (secondary cultivation substrate) to produce mushrooms from another fungus.

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The secondary cultivation substrate (2 kg) was prepared from the spent mushroom substrate (SMS) of *L. crinitus* (SMSLC), *P. ostreatus* (SMSPO), or *P. djamor* (SMSPD) with the addition of SB to adjust the C/N ratio between 46 and 50, growth range of *Pleurotus* and *Lentinus*. The secondary cultivation substrate was inoculated with a fungus different from the one used in the previous cultivation. Four experiments were established based on the reuse of SMS to investigate microbial succession with two fungi of different species. *P. ostreatus* mushrooms were produced in SMS from the primary cultivation of *L. crinitus*, and *L. crinitus* was cultivated in SMS from *P. ostreatus*. Similarly, *P. djamor* mushrooms were grown on SMS from *L. crinitus*, and *L. crinitus* cultivated on SMS from *P. djamor*.

2.4 Mushroom harvesting and production analysis

After colonizing the primary or secondary cultivation substrate, the upper part of the plastic bag was removed, and the room ventilation was activated, keeping the relative humidity at 80%. The mushroom harvest occurred when the edge of the pileus was open, indicating the beginning of the senescence process. Mushroom productivity (wet basis) of each cultivation substrate (2 kg; wet basis) was recorded over time, and biological efficiency was determined at the end of the production period. Biological efficiency was calculated by the ratio between the fresh mushroom mass and the substrate dry mass, multiplied by 100 (Chang and Miles, 2004). The mushrooms were dehydrated in an oven with air circulation at 60 °C until constant mass, and subsequently, grounded after freezing with nitrogen and stored at -20 °C.

2.5 Basidiocarps and substrates extracts preparation

The methanolic extract was analyzed to determine the antioxidant activity by the DPPH method (2,2-diphenyl-1-picrylhydrazyl) (Sánchez-Moreno *et al.*, 1998) and by the beta-carotene/linoleic acid (BCLA) co-oxidation system (Mattos *et al.*, 2009). In addition, the aqueous extract was analyzed for antioxidant activity by the FRAP (ferric reducing antioxidant power) method (Rufino *et al.*, 2006). A sample of 1 g of the grounded fruiting body or 1 g of the substrate (dry basis) was mixed with

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10-mL methyl alcohol or 10-mL ultrapure water. The mixtures were incubated at 60 ˚C for 45 min, centrifuged at 6000 *g* at 5 ˚C for 10 min, and the supernatant was used for the antioxidant activity determination (Bertéli *et al.*, 2021a).

2.6 Antioxidant activity

2.6.1 DPPH radical-scavenging assay

The antioxidant activity by the DPPH method was determined according to Bertéli *et al.* (2021a). Aliquots (8 µL) of the fruiting bodies or substrates extracts (20, 40, 60, 80, and 100 mg mL^{-1}) were mixed with 292 µL of methanolic DPPH solution (60 µM). Methanol (300 µL) was used as a negative control. After 30 min, the absorbance was determined at 515 nm (Spectramax Plus 384 spectrophotometer). The total antioxidant capacity of the extract was calculated using a standard solution of quercetin (60 µM) as a 100% reference (Sánchez-Moreno, 1998). The sample concentration needed to reduce 50% of free radicals (IC_{50}) was determined by a correlation between absorbance and sample concentration. All assays were performed in three repetitions.

2.6.2 Ferric reducing antioxidant power (FRAP) assay

The analysis of antioxidant activity by the FRAP method was performed with a mixture of 290 µL of FRAP reagent (2.5 mL of 10 mM 2,4,6-tris (2-pyridyl)-s-triazine, 25 mL of 0.3 M acetate buffer, and 2.5 mL of 20 mM ferric chloride) and 10-µL extract. The mixtures were homogenized, kept at 37 °C for 30 min, and the absorbance was determined at 595 nm (Spectramax Plus 384 spectrophotometer). Antioxidant activity was calculated with a standard curve of ferrous sulfate (2000 µM). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as positive control (Rufino *et al.*, 2006). All assays were performed in three repetitions.

2.6.3 β-carotene/linoleic acid (BCLA) assay

The antioxidant activity of the fruiting body and substrates extracts by the BCLA method was performed according to Mattos *et al.* (2009). Aliquots (20 µL) of

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the extracts (100 mg mL^{-1}) were mixed with 280 µL of the system solution (β-carotene/linoleic acid). The mixture was incubated at 37 °C, and the absorbance was determined at 470 nm after 2 min and then every 15 min for 120 min (Spectramax Plus 384 spectrophotometer). Antioxidant activity was expressed as a percentage of inhibition of co-oxidation. Trolox was used as a positive control. The reduction in absorbance of the system without antioxidants was considered 100% oxidation.

2.7 Statistical analysis

The assays were carried out in a completely randomized design. The arithmetic means and standard deviations were calculated from independent growing bags, with ten repetitions for productivity and biological efficiency and three for antioxidant activity, there were no contaminations. Data were analyzed by analysis of variance (ANOVA) and was used because it compared the means of three or more groups to check if at least one mean differed and differences between arithmetic means determined by Tukey's test, for pairwise comparisons after the ANOVA has indicated differences, keeping the overall error under control or Student test or Student's unpaired t-test, which compares means of two independent groups ($p \leq 0.05$) using statistical software (IBM SPSS 22).

3. Results

The necessary amounts of SB and SM to formulate the primary and secondary cultivation substrates were calculated based on the C/N ratio of agro-industrial by-products and spent substrates (Table 1).

Table 1. Proximate composition of agro-industrial by-products and spent mushroom substrate (SMS) from *Lentinus crinitus*, *Pleurotus ostreatus*, and *Pleurotus djamor*.

Material	Carbon (%)	Nitrogen (%)	Moisture (%)	Ashes (%)	C/N ratio
Sugarcane bagasse	34.81	0.42	24.76	5.62	82.88
Soybean meal	39.61	7.14	15.07	5.71	5.55

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SMSLC	40.64	1.35	15.07	7.95	30.14
SMSPO	41.63	2.05	11.55	4.59	20.32
SMSPD	41.59	1.21	13.20	3.15	34.27

Average values on a wet basis; C/N = carbon/nitrogen; SMSLC = spent mushroom substrate of *Lentinus crinitus*; SMSPO = spent mushroom substrate of *Pleurotus ostreatus*; SMSPD = spent mushroom substrate of *Pleurotus djamor*.

As a result, the C/N ratio of all cultivation substrates ranged from 46.2 to 50.9, with a mean of 47.7 ± 2.1 (Table 2).

Table 2. The substrates' carbon/nitrogen ratio (C/N) for *Lentinus crinitus*, *Pleurotus ostreatus*, and *Pleurotus djamor* cultivation.

Cultivated fungus	Cultivation substrate	Proportion (%)	C/N ratio
Lentinus crinitus, *Pleurotus ostreatus*, *Pleurotus djamor*	SB:SM	95:5	46.36
Lentinus crinitus	SB:SMSPO	77.5:22.5	46.19
Lentinus crinitus	SB:SMSPD	60:40	50.98
Pleurotus ostreatus	SB:SMSLC	60:40	46.89
Pleurotus djamor	SB:SMSLC	60:40	46.89

SB = Sugarcane bagasse; SM = Soybean meal; SMSLC = spent mushroom substrate of *Lentinus crinitus*; SMSPO = spent mushroom substrate of *Pleurotus ostreatus*; SMSPD = spent mushroom substrate of *Pleurotus djamor*.

The spent substrate from primary cultivation showed a mass reduction of 62.45 ± 10.89% to 81.19 ± 4.40%, with the most significant mass substrate reduction produced by the cultivation of *Pleurotus* (Table 3). On the other hand, the degradation of the secondary cultivation substrate was lower than in the primary cultivation varying from 36.24 ± 6.81% to 55.08 ± 9.42% (Table 3).

Table 3. Reduction of cultivation substrates masses (dry basis) after 80 days of *Lentinus crinitus, Pleurotus ostreatus,* and *Pleurotus djamor* cultivation.

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Cultivation substrates	Initial mass (g)	Final mass (g)	Substrate mass reduction (%)
Primary SMSLC (SB:SM)	440	165.21 ± 47.92^{c}	62.45 ± 10.89^{b}
Primary SMSPO (SB:SM)	440	82.75 ± 19.38^{e}	81.19 ± 4.40^{a}
Primary SMSPD (SB:SM)	440	92.12 ± 20.29^{d}	79.06 ± 4.61^{a}
Secondary SMSLC (SB:SMSPO)	440	197.64 ± 41.46^{b}	55.08 ± 9.42^{c}
Secondary SMSLC (SB:SMSPD)	440	205.16 ± 36.84^{b}	53.37 ± 8.37^{c}
Secondary SMSPO (SB:SMSLC)	440	278.74 ± 23.69^{a}	36.65 ± 5.38^{d}
Secondary SMSPD (SB:SMSLC)	440	280.75 ± 29.99^{a}	36.24 ± 6.81^{d}

Arithmetic means ± standard deviation. Means with different letters in the same column differ significantly by Tukey's test ($p \leq 0.05$). SB = sugarcane bagasse; SM = Soybean meal; SMSLC = spent mushroom substrate of *Lentinus crinitus*; SMSPO = spent mushroom substrate of *Pleurotus ostreatus*; SMSPD = spent mushroom substrate of *Pleurotus djamor*.

The productivity and biological efficiency (BE) of mushrooms in primary cultivation were greater than in secondary cultivation. In primary cultivation, the highest mushroom yields were of *P. ostreatus* and *P. djamor* (statistically equal), with 437.70 ± 86.84 g and 417.70 ± 48.27 g, respectively, followed by *L. crinitus* with the production of 53.19 ± 28.77 g (Table 4). In secondary cultivation, there was a significant reduction in productivity. Cultivation in SMSLC reduced the productivity of *P. djamor* by 4.4 times and that of *P. ostreatus* by 4.2 times. The secondary cultivation of *L. crinitus* mushrooms in the substrate with SMSPD reduced the mushroom productivity 1.4-fold, but in SMSPO, the productivity of *L. crinitus* remained unchanged (Table 4). The primary cultivation of *P. ostreatus* and *P. djamor* presented BE above 90%; meanwhile, *L. crinitus* showed only 12%. However, in secondary cultivation using SMS, the BE of *Pleurotus* sp. was less than 27%, and *L. crinitus* cultivated in SMSPD was under 9% (Table 4).

Table 4. Mushroom productivity (g) per cultivation substrate (2 kg; wet basis) and biological efficiency (BE) after 80 days of cultivation of *Lentinus crinitus* (LC), *Pleurotus ostreatus* (PO), and *Pleurotus djamor* (PD).

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Primary cultivation			Secondary cultivation			
Fungus	Mushroom productivity (g)	BE (%)	Fungus	Mushroom productivity (g)	BE (%)	*p*-value
LC	53.19 ± 28.77[b]	12.09 ± 6.54[b]	LC (SB:SMSPO)	68.71 ± 31.44[b]	15.62 ± 7.15[b]	0.121
LC	53.19 ± 28.77[b]	12.09 ± 6.54[b]	LC (SB:SMSPD)	39.28 ± 10.29[c]	8.92 ± 2.34[c]	0.038
PO	437.70 ± 86.84[a]	99.48 ± 19.74[a]	PO (SB:SMSLC)	105.33 ± 22.60[a]	26.33 ± 5.65[a]	<0.001
PD	417.70 ± 48.27[a]	94.94 ± 10.97[a]	PD (SB:SMSLC)	93.89 ± 24.64[a]	23.47 ± 6.16[a]	<0.001

Arithmetic mean ± standard deviation. Means with different letters in the same column differ significantly by Tukey's test ($p \leq 0.05$). *P*-values in the lines indicate significant differences in mushroom productivity in primary and secondary cultivation substrates by Student's t-test ($p \leq 0.05$; n= 10). SB = Sugarcane bagasse; SMSLC = spent mushroom substrate of *Lentinus crinitus*; SMSPO = spent mushroom substrate of *Pleurotus ostreatus*; SMSPD = spent mushroom substrate of *Pleurotus djamor*.

The antioxidant activity of SMS determined by the DPPH method was lower than the quercetin control. However, there was an increase in the antioxidant activity of all substrates after microbial succession (Table 5). The most significant increase in antioxidant activity was observed in SMS when *L. crinitus* succeeded *P. djamor* in the secondary cultivation where the IC_{50} reduced from 463.06 ± 34.82 mg mL^{-1} to 254.33 ± 1.94 mg mL^{-1} (Table 5). The reduction in SMS' IC_{50} was also significant in secondary cultures where *L. crinitus* succeeded *P. ostreatus* (1.5-fold lower), and *Pleurotus* sp. succeeded *L. crinitus* (both, 1.2-fold lower) (Table 5).

Lentinus crinitus and *P. djamor* mushrooms produced in the primary cultivation showed statistically similar antioxidant activities determined by DPPH radical scavenging assay (IC_{50} ~ 240 mg mL^{-1}) (Table 5). In contrast, the antioxidant activity of *P. ostreatus* was lower (IC_{50} 1.0-fold higher). Mushrooms produced in secondary cultivation showed higher antioxidant activity than in primary cultivation, except for *P. djamor* mushrooms, whose antioxidant activity was the same in both cultivations (Table 5). Mushrooms of *L. crinitus* produced in succession to *P. djamor*

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had the highest anti-DPPH activity (IC_{50} = 94.39 ± 0.90 mg mL^{-1}), with a 2.5-fold reduction in IC_{50} compared to mushrooms from the primary cultivation. Mushrooms of *L. crinitus* produced in SMSPO and *P. ostreatus* grown in SMSLC also showed an increase in anti-DPPH activity with a 2.3-fold and 1.7-fold reduction, respectively, in the IC_{50} (Table 5).

Table 5. Antioxidant activity of *Lentinus crinitus* (LC), *Pleurotus ostreatus* (PO), and *Pleurotus djamor* (PD) mushrooms produced in primary and secondary cultivation and of the respective spent mushroom substrates by 2.2-diphenyl-1-picrylhydrazyl (DPPH; IC_{50}) free radical reduction method.

Spent substrate from primary cultivation		Spent substrate from secondary cultivation			Mushroom from primary cultivation		Mushroom from secondary cultivation		
Substrate	IC_{50} (mg mL^{-1})	Substrate	IC_{50} (mg mL^{-1})	*p*-value	Mushroom	IC_{50} (mg mL^{-1})	Mushroom	IC_{50} (mg mL^{-1})	*p*-value
SMSLC	463.06 ± 34.82^{c}	SMSLC (SB:SMSPO)	314.98 ± 9.59^{c}	0.004	LC	231.71 ± 7.73^{a}	LC (SB:SMSPO)	101.53 ± 2.45^{b}	<0.001
SMSLC	463.06 ± 34.82^{c}	SMSLC (SB:SMSPD)	254.33 ± 1.94^{b}	0.009	LC	231.71 ± 7.73^{a}	LC (SB:SMSPD)	94.39 ± 0.90^{a}	<0.001
SMSPO	258.42 ± 25.54^{a}	SMSPO (SB:SMSLC)	209.84 ± 13.82^{a}	0.040	PO	258.42 ± 11.10^{b}	PO (SB:SMSLC)	147.78 ± 8.49^{c}	0.003
SMSPD	326.78 ± 3.20^{b}	SMSPD (SB:SMSLC)	265.86± 11.91^{b}	0.002	PD	240.46 ± 5.30^{a}	PD (SB:SMSLC)	226.97 ± 18.40^{d}	0.202

Arithmetic mean ± standard deviation. Means with different letters in the same column differ significantly by Tukey's test ($p \leq 0.05$). *P*-values in the lines indicate significant differences in antioxidant activity in the spent substrates and mushrooms from primary and secondary cultivation by Student's t-test ($p \leq 0.05$; n= 3). SB = Sugarcane bagasse; SMSLC = spent mushroom substrate of *Lentinus crinitus*; SMSPO = spent mushroom substrate of *Pleurotus ostreatus*; SMSPD = spent mushroom substrate of *Pleurotus djamor*. Quercetin reference control = 0.0155 ± 0.10 mg mL^{-1}.

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The substrates of primary cultivation of *P. ostreatus* and *P. djamor* exhibited the highest antioxidant activities by the FRAP method, 15.73 ± 0.78 µmol Fe^{+2} g^{-1} and 12.00 ± 0.63 µmol Fe^{+2} g^{-1}, respectively (Table 6). These activities were higher than the Trolox positive control activity (1.5-fold and 1.2-fold greater). However, substrates of the secondary cultivation of all mushrooms showed lower antioxidant activity by FRAP than the primary cultivation. When *P. ostreatus* succeeded *L. crinitus*, the FRAP activity of the SMS was 3.0 times lower, and when *P. djamor* followed *L. crinitus*, the activity was 2.0 times lower. In the substrate where *L. crinitus* was cultivated, there was also a significant reduction in antioxidant activity, 1.7 times in SMSPO and 1.5 times in SMSPD (Table 6).

When the antioxidant activity by FRAP of mushrooms from the primary cultivation was evaluated, *P. djamor* (27.54 ± 1.74 µmol Fe^{+2} g^{-1}) had the most significant capacity to reduce iron, 2.6-fold higher than the Trolox. Mushrooms of *L. crinitus* (21.25 ± 0.31 µmol Fe^{+2} g^{-1}) and *P. ostreatus* (19.37 ± 0.59 µmol Fe^{+2} g^{-1}) also exhibited high antioxidant activity, two times and 1.8 times higher than Trolox (Table 6). However, succession cultivation increased the antioxidant activity by FRAP of the mushrooms, except for *P. djamor* cultivated in SMSLC whose activity was lower than in the primary cultivation but still twice as much as Trolox. Mushrooms of *L. crinitus* grown in SMSPD (75.51 ± 1.98 µmol Fe^{+2} g^{-1}), and SMSPO (66.01 ± 4.59 µmol Fe^{+2} g^{-1}) showed the highest antioxidant activities by this method, seven times and 6.3 times superior to Trolox. Compared to the antioxidant activities of *L. crinitus* mushrooms from primary cultivation, the FRAP activity of the mushrooms in succession more than tripled (Table 6). Mushrooms of *P. ostreatus* that succeeded *L. crinitus* in the cultivation also showed higher antioxidant activity than the primary cultivation (2.8-fold higher) and five times greater than the control.

Table 6. Antioxidant activity of *Lentinus crinitus* (LC), *Pleurotus ostreatus* (PO), and *Pleurotus djamor* (PD) mushrooms produced in primary and secondary cultivation and of the respective mushroom substrates by the ferric reducing antioxidant power (FRAP) assay.

Spent substrate from primary	Spent substrate from secondary cultivation	Mushroom from primary	Mushroom from secondary cultivation

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cultivation					cultivation				
Substrate	FRAP (µmol Fe^{+2} g^{-1})	Substrate	FRAP (µmol Fe^{+2} g^{-1})	*p*-value	Mushroom	FRAP (µmol Fe^{+2} g^{-1})	Mushroom	FRAP (µmol Fe^{+2} g^{-1})	*p*-value
SMSLC	8.07 ± 0.55^{b}	SMSLC (SB:SMSPO)	4.62 ± 0.15^{a}	<0.001	LC	21.25 ± 0.31^{b}	LC (SB:SMSPO)	66.01 ± 4.59^{b}	0.002
SMSLC	8.07 ± 0.55^{b}	SMSLC (SB:SMSPD)	5.37 ± 0.07^{a}	0.006	LC	21.25 ± 0.31^{b}	LC (SB:SMSPD)	75.51 ± 1.98^{a}	<0.001
SMSPO	15.73 ± 0.78^{a}	SMSPO (SB:SMSLC)	5.39 ± 0.17^{a}	<0.001	PO	19.37 ± 0.59^{b}	PO (SB:SMSLC)	53.63 ± 2.99^{c}	<0.001
SMSPD	12.00 ± 0.63^{a}	SMSPD (SB: SMSLC)	5.70 ± 0.15^{a}	<0.001	PD	27.54 ± 1.74^{a}	PD (SB:SMSLC)	19.65 ± 0.69^{d}	<0.001

Arithmetic mean ± standard deviation. Means with different letters in the same column differ significantly by Tukey's test ($p \leq 0.05$). *P*-values in the lines indicate significant differences in antioxidant activity in the spent substrates and mushrooms from primary and secondary cultivation by Student's t-test ($p \leq 0.05$; n= 3). SB = Sugarcane bagasse; SMSLC = spent mushroom substrate of *Lentinus crinitus*; SMSPO = spent mushroom substrate of *Pleurotus ostreatus*; SMSPD = spent mushroom substrate of *Pleurotus djamor*. Trolox reference control = 10.5 ± 0.9 µmol Fe^{+2} g^{-1}.

The antioxidant activities of SMS from primary and secondary cultures determined by the β-carotene bleaching inhibition method (BCLA) were not expressive, and all were below 27% (Table 7). The highest BCLA activities of primary cultivation SMS were observed in *L. crinitus* and *P. ostreatus* SMS (26.22% and 24.09%, respectively), but *P. djamor* SMS inhibited β-carotene bleaching only 16.36 ± 5.18 % (Table 7). Among the antioxidant activities of secondary culture SMS, the highest were those where *L. crinitus* succeeded *P. djamor* (20.83 ± 14.08%) and where *P. djamor* succeeded *L. crinitus* (19.11 ± 11.28%). However, the microbial succession reduced the antioxidant activity by BCLA in all treatments, but significantly only in the substrate where *L. crinitus* succeeded *P. ostreatus* (2.0 times lower) and where *P. ostreatus* succeeded *L. crinitus* (1.3 times lower).

Mushrooms of *P. djamor* and *P. ostreatus* produced in the primary cultivation showed the highest inhibitions of β-carotene bleaching, statistically similar, with 85.48 ± 14.49% and 76.44 ± 14.32%. Mushrooms of *L. crinitus* presented 63.55 ±

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10.29% inhibition (Table 7). The antioxidant activity by BCLA of *P. ostreatus* mushrooms grown in microbial succession was 96.29 ± 4.10 %, 1.1 times higher than the positive control Trolox and significantly higher than the activity of mushrooms from the primary cultivation. Mushrooms of *L. crinitus* produced in succession to *P. djamor* and *P. djamor* in sequence to *L. crinitus* inhibited β-carotene bleaching by 74.62 ± 4.67% and 81.00 ± 12.71 %, respectively. However, the activity was not significantly different from mushrooms produced in primary cultivation.

Table 7. Antioxidant activity of *Lentinus crinitus* (LC), *Pleurotus ostreatus* (PO), and *Pleurotus djamor* (PD) mushrooms produced in primary and secondary cultivation and of the respective mushroom substrates by the β-carotene/linoleic acid (BCLA) assay.

Spent substrate from primary cultivation		Spent substrate from secondary cultivation			Mushroom from primary cultivation		Mushroom from secondary cultivation		
Substrate	BCLA (%)	Substrate	BCLA (%)	*p*-value	Mushroom	BCLA (%)	Mushroom	BCLA (%)	*p*-value
SMSLC	26.22 ± 3.93[a]	SMSLC (SB:SMSPO)	13.32 ± 5.07[b]	0.013	LC	63.55 ± 10.29[b]	LC (SB:SMSPO)	69.45 ± 9.82[c]	0.256
SMSLC	26.22 ± 3.93[a]	SMSLC (SB:SMSPD)	20.83 ± 14.08[a]	0.279	LC	63.55 ± 10.29[b]	LC (SB:SMSPD)	74.62 ± 4.67[b]	0.082
SMSPO	24.09 ± 5.53[a]	SMSPO (SB:SMSLC)	14.58 ± 3.46[b]	0.032	PO	76.44 ± 14.32[a]	PO (SB:SMSLC)	96.29 ± 4.10[a]	0.041
SMSPD	16.36 ± 5.18[b]	SMSPD (SB:SMSLC)	19.11 ± 11.28[a]	0.360	PD	85.48 ± 14.49[a]	PD (SB:SMSLC)	81.00 ± 12.71[b]	0.354

Arithmetic mean ± standard deviation. Means with different letters in the same column differ significantly by Tukey's test ($p \leq 0.05$). *P*-values in the lines indicate significant differences in antioxidant activity in the spent substrates and mushrooms from primary and secondary cultivation by Student's t-test ($p \leq 0.05$; n= 3). SB = Sugarcane bagasse; SMSLC = spent mushroom substrate of *Lentinus crinitus*; SMSPO = spent mushroom substrate of *Pleurotus ostreatus*; SMSPD = spent mushroom substrate of *Pleurotus djamor*. BCLA (% bleaching inhibition). Trolox reference control = 85.6 ± 1.0%.

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4. Discussion

The concentration of nitrogen and the substrate's carbon/nitrogen ratio (C/N) are fundamental for producing mushrooms, as they are essential in the metabolism and constitution of the fungal cell wall (Chang and Miles, 2004). Therefore, the imbalance of nitrogen content in the cultivation substrate is one of the most critical factors affecting the growth of mycelial biomass, which may cause its inhibition (Dekker *et al.*, 2007). Previous reports assumed that the desirable C/N ratio for basidiomycetes axenic cultivation and mushroom production is between 20 and 50 (Eira, 2004).

Our results indicated a reduction in the productivity of mushrooms and BE when the cultivation occurred in substrates containing SMS. Siqueira *et al.* (2016) reported that the decline in *P. ostreatus* productivity was negatively correlated with the percentage of *P. ostreatus* SMS in the cultivation substrate. Wang *et al.* (2015) reported that the addition of more than 25% of SMS of *Hypsizigus marmoreus* resulted in a decreased yield of *P. ostreatus* mushrooms.

Lisiecka *et al.* (2021) also observed that increasing the amount of SMS from *Hericium erinaceus* and *Pholiota nameko* above 30% in the substrate decreased the yield of *P. ostreatus* mushrooms. In our study, we set the SMS added at 40% to obtain an adequate C/N ratio. The only exception was preparing the *L. crinitus* cultivation substrate in SMSPO, where 22.5% of SMS were added to the substrate. It is noteworthy that in secondary cultivation, only the yield of *L. crinitus* in SMSPO did not decrease, while in the other treatments, the yield decreased significantly.

The BE values obtained in the present study showed wide variation, and *L. crinitus* always presented BE lower than *P. ostreatus* and *P. djamor.* The BE in secondary cultivation (8.92% to 26.33%) was lower than in primary cultivation (12.09% to 99.48%), following the trend of decreasing productivity. Picornell *et al.* (2015) also reported a wide range of BE (2.77% to 58.48%) when *P. ostreatus* was produced in a substrate with the SMS of *P. ostreatus*. Siqueira *et al.* (2016), who grew *P. ostreatus* in SMS from *P. ostreatus*, also reported wide variation in BE (3.85% to 42.87%). On the other hand, Lisiecka *et al.* (2021) cultivated *P. ostreatus*

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on SMS from *H. erinaceus*, and *P. nameko* reported minor variation in BE (66.48% to 72.67%), even the substrates containing different percentages of SMS.

The reduction in productivity and BE during secondary cultivation may result from substrate modifications after primary cultivation, such as changes in the physicochemical composition of substrates and the presence of elements produced by the fungus's metabolism during growth (Almeida *et al.*, 2019). Increased nutrient availability or substrate impoverishment after primary cultivation can decrease productivity and BE during mushroom production in microbial succession. In a study with *P. florida*, the amount of nutrients affected the growth of *P. florida* (Figueiro and Graciolli, 2011). Additionally, the lignin and hemicellulose form a physical barrier that hinders the activity of fungal cellulolytic enzymes, restricting the attack on the external surface of the substrate (Figueiro and Graciolli, 2011). The results indicate that physicochemical factors may have been decisive in reducing productivity and BE.

The composition of the substrate, the cultivation environment and the fungus species influence the chemical composition of the mushrooms and, consequently, the biological activity of the basidiomes (Braga *et al.*, 1998). Furthermore, studies have shown that bioactive compounds produced during growth permeate the cultivation substrate (Mizuno *et al.*, 1999; He *et al.*, 2016; Sardar *et al.*, 2018) adding value to SMS. Mushrooms have bioactive compounds considered valuable natural antioxidants, a property attributed to different compounds such as carotenoids, phenolic compounds, tocopherols, organic acids, and polysaccharides (Boonsong *et al.*, 2016). Phenolic compounds and tocopherols have been the most widely investigated antioxidants in mushrooms (Palacios *et al.*, 2011).

The chemical composition and bioactive compounds of *L. crinitus* mushrooms and their SMS are still poorly known since this species is not industrially produced. Bertéli *et al.* (2021a) recently demonstrated that the basidiocarp of this fungus is rich in β-tocopherol, phenolic compounds (particularly *p*-hydroxybenzoic acid), and organic acids. Also, *L. crinitus* basidiocarp presented a broad antimicrobial activity against different species of bacteria and fungi. *L. crinitus* mushrooms were produced in primary cultivation in a substrate with sugarcane

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bagasse and rice husk (Bertéli *et al.*, 2021b). The pileus had higher levels of protein, tocopherols, and malic acid, while the stipe had higher contents of carbohydrates, energy, soluble sugars, and *p*-hydroxybenzoic acid. The mushrooms also had high antioxidant activity by the FRAP and BCLA methods with higher activities of the pileus than stipe. These findings are very similar to our study. Our results showed that basidiocarps of *L. crinitus* in secondary cultivation showed higher activity by the FRAP method than that described by Bertéli *et al.* (2021a). When *L. crinitus* was cultivated in a substrate with SMSPO and SMSPD, the activity by FRAP was 1.9-fold and 2.1-fold higher, respectively, than the pileus in Bertéli *et al.* (2021a). Similar results were observed with the BCLA method. The basidiocarps produced in our study by primary cultivation exhibited 63.55% inhibition of β-carotene oxidation, an effect similar to those reported (~56%) by Bertéli *et al.* (2021a). However, when basidiocarps were produced in substrates with SMSPO and SMSPD, the antioxidant activity by BCLA increased, surpassing 1.2 times and 1.3 times, respectively, the activity reported by Bertéli *et al.* (2021a).

The antioxidant activity of *P. djamor* and *P. ostreatus* has already been reported. The mushrooms of both showed antioxidant activity by FRAP and BCLA and low activity by the DPPH method. The highest values found in our study by the DPPH antioxidant method were for *P. djamor* and *P. ostreatus* grown on secondary substrate SMSLC. However, the results were lower than those found in the literature, being five times lower than reported by Stojkovic *et al.* (2014), 9-15 times smaller than in Yilmaz *et al.* (2017), 26 times smaller than in Reis *et al.* (2012), a fact that indicates low antioxidant activity by DPPH of mushrooms, both in the primary substrate and in SMS. The basidiocarps of *P. ostreatus* produced in primary and secondary cultivation showed greater activity by the FRAP method than Yilmaz *et al.* (2017). The FRAP values of *P. djamor* cultivated in the primary substrate and substrate containing SMSLC were 2.2 and 1.6 times higher, respectively than those reported by Yilmaz *et al.* (2017). *P. ostreatus* cultivated in the primary substrate and SMSLC showed FRAP activity was 1.6 and 4.3 times higher, respectively than the highest value found for *P. ostreatus* by Yilmaz *et al.* (2017). The basidiocarps of *P. djamor* produced in our study by primary and secondary cultivation (SMSLC)

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exhibited 85.5% and 81% inhibition of β-carotene oxidation, respectively, and the *P. ostreatus* mushroom produced in the primary substrate 76.44%. These findings indicate values lower than or similar to the maximum value reported (95.39%) by Kanagasabapathy *et al.* (2011) for *Pleurotus sajor-caju*. However, when the basidiocarp of *P. ostreatus* was produced in the substrate with SMSLC, the antioxidant activity by BCLA increased (96.29%), surpassing the percentage reported by Kanagasabapathy *et al.* (2011).

Corrêa *et al.* (2015) demonstrated that the basidiocarp of *P. djamor* is rich in phenolic compounds (*p*-hydroxybenzoic acid), tocopherols (β-tocopherol), and organic acids, mainly citric and malic. Furthermore, *P. djamor* showed significant anti-inflammatory and antimicrobial potential against different species of bacteria and fungi. *P. ostreatus* is a recognized functional food with high fiber and protein content and low carbohydrate and fat content (Salata *et al.*, 2018). Its basidiocarp is rich in phenolic compounds, mainly *p*-hydroxybenzoic acid (Muszynska *et al.*, 2013), has anti-inflammatory, antibacterial, and antifungal potential (Jedinak *et al.*, 2011), in addition to antitumor properties (Maiti *et al.*, 2011). Thus, the bioactive compounds and metabolites produced by the growing mycelium possibly permeated the cultivation substrate, influencing the antioxidant activity of mushrooms from the secondary cultivation and of the substrate itself.

4.1 Limitations

This work requires a significant amount of waste, which limits the number of independent replicates; that is, a superstructure is needed for large-scale mushroom cultivation, which would allow for an increase in the number of replicates of independent bags. Nutritional and chemical analyses can provide further support for bolder conclusions.

5. Conclusions

The succession process of mushroom, in general, decreases mushroom production and biological efficiency and promotes a decrease in substrate consumption.

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The antioxidant activity by DPPH radical sequestration from the secondary culture substrate increased compared to the primary culture substrate. However, the antioxidant activity by FRAP and BCLA of the secondary substrate decreased compared to the primary culture substrate. Except for the secondary cultivation substrate (SMSLC) of *P. djamor*, which presented BCLA activity 1.2 times higher than the primary substrate.

The antioxidant activity of mushrooms by the DPPH method increased by 2.5 times in *L. crinitus* grown in SMSPD (94.39 ± 0.90 mg mL^{-1}). There was a 3.6-fold increase in the antioxidant activity of mushrooms by the FRAP method of secondary cultivation of *L. crinitus* produced in SMSPD (75.51 ± 1.98 µmol Fe^{+2} g^{-1}). By BCLA, the antioxidant activity of *P. ostreatus* mushrooms from secondary cultivation was 1.3 times higher (96.29 ± 4.10%) than their primary counterpart.

Mushrooms show remarkable antioxidant activity by the FRAP method, with all results superior to the reference control Trolox. The highest activity is of *L. crinitus* from secondary succession grown in SMSPD with a value 7.2 times higher than Trolox. Therefore, SRC induced an increase in antioxidant activity.

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