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Effects of Incubation Time and "Browning" on Yield and Proximate Composition of the Edible Mushroom *Lentinula edodes*

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HIGHLIGHTS

- Induction of browning and incubation time do not affect Shiitake proximate composition.
- Induction of browning does not affect Shiitake biological efficiency.
- The strain used changes Shiitake proximate composition.
- The strain used and incubation time affect Shiitake biological efficiency.

Abstract: *Lentinula edodes* is the most consumed mushroom in the world, being cultivated mainly on waste coming from the forest industry. During shiitake cultivation, incubation is essential and is usually longer than in other edible mushrooms. This stage includes "browning", which is a process induced by exposure to a photoperiod and although it is believed to bring certain advantages to shiitake cultivation, it has not been widely studied. In this work, we evaluated how the incubation time, the use of different strains and the induction of browning affect the proximate composition, biological efficiency and other yield parameters of *L. edodes*. In order to do that, three experiments were carried out with three different strains, three different incubation times and the effect of induction or non-induction of browning. As results we found that the proximate composition did not vary with respect to the different incubation times or from the induction of browning, although differences were found according to the strain. On the other hand, the biological efficiency was affected by the incubation time and the strain used, but not by the induction of browning.

Keywords: food production; mushroom cultivation; shiitake; biological efficiency.

INTRODUCTION

Mushrooms have the ability to convert lignocellulosic materials such as those coming from the forest industry into human food. In general, they have short growing periods, very low input requirements for production, simple production technologies, and independence from weather conditions [1]. Edible mushrooms represent an alternative source of important nutrients such as carbohydrates, proteins, vitamins, and minerals, among others [2], and their nutritional composition is comparable to eggs, milk and meat [3]. From this point of view, mushrooms are healthy food that have been part of the positive effect on human well-being called the "non-green revolution" that generates equitable economic growth and reduces environmental pollution by regenerating valuable resources [4,5].

The shiitake mushroom, *Lentinula edodes*, is the most cultivated mushroom in the world, contributing about 22% of the world's mushrooms production [6]. It can be used as food and as medicine and has served as a model for investigating functional properties of fungi and for isolating pure compounds for pharmaceutical use [7]. *L. edodes* is a rich source of carbohydrates and proteins since it contains 18 types of free amino acids which provide ideal ratios of all the essential amino acids needed for human nutrition [8]. Quality and content of proximate composition and physiologically active substances vary from strain to strain [9,10], and also depend on substrate, supplementation [7,11,12], culture and growth conditions [13], or postharvest processes of fruiting bodies [14,15]. However, there is no detailed information about how some steps of the cultivation process affect the proximate composition. Shiitake has been traditionally cultivated on natural logs, but this resulted to be an unsustainable method for a high production scale since the time required for growing trees is longer than the cultivation cycle on synthetic logs. This method was replaced mainly by the cultivation on synthetic logs, based on autoclaving sawdust. In addition, alternative production techniques have been tested, in order to reduce production costs, using pasteurization as heat treatment or cereal straw as substrates [16, 17, 18, 19]. Within the productive process, incubation is one of the most important phases for shiitake cultivation [20]. For most mushrooms it is common to achieve full colonization in 20 days and then begin the fructification phase immediately. *L. edodes* is the one mushroom that according to common practices and literature, needs a larger period of incubation. Incubation of shiitake includes different processes: 1) colonization: the growth of mycelium through the substrate that usually takes 30 days; 2) lumping: production of relatively large (0.5-2 cm) lumps protruding from the surface of the block; 3) browning: the production of a brown, skin-like surface on the block that can last up to 30 days and 4) pinning: the production of mushroom primordia [21].

Browning is considered an important step for shiitake production and it is performed routinely by most shiitake growers; this process is induced through the exposure of the mycelium blocks to a photoperiod during the incubation phase. Some studies have shown that intensity of browning can vary according to light, aeration and temperature [22,23]; the process is accelerated by oxygen and light exposition [24]. Some enzymatic activities are modified: enzymes related to light sensing (kinases and G proteins-coupled receptors), melanogenesis (tyrosinases) and cell wall degradation (glucanases, chitinases, laccases, manganese peroxidase and lignin peroxidase) [25,26]. The browning process develops a brown layer of mycelia which not only improves mushroom quality but also discourages mold contamination [27]. However, the real implication of this process on the biological efficiency and other performance parameters such as the proximate composition of the harvested fruiting bodies, is yet unknown. The aim of this work is to evaluate how the incubation time and the induction of browning affect the proximate composition, the biological efficiency and the yield parameters of the fruiting bodies of *L. edodes*.

MATERIAL AND METHODS

Strains

Three commercial strains of shiitake were used: ICFC 510/03, ICFC 879/17 and ICFC 293/00. These strains are commonly used by local mushroom farmers and they are conserved in the INTECH Collection of Fungal Cultures (ICFC) of the Laboratory of Mycology and Mushroom Cultivation in Chascomús, Argentina; reference in the WDCM database: 826.

Spawn production

Spawn was prepared in plastic bags containing 500 g of boiled oat grains and 2% w/w CaCO3, then the bags were sterilized during 2.5 h at 121 °C, cooled at room temperature and inoculated with an agar plug (1 cm diam.) with actively growing mycelium of the selected strain. Bags were incubated in the dark at 25 °C and mixed gently by hand periodically during 21 days.

Substrate preparation and spawning

Substrate formulation (on a dry basis) consisted of: 40% *Eucalyptus* sawdust, 40% *Eucalyptus* chips, 16.75% wheat bran and 3.25% gypsum ($CaSO_4$). All the ingredients were mixed and the relative humidity was adjusted to 65%. Mixed substrate was bagged into 2 kg bags with a filter patch and then sterilized at 121 ºC for 2 h. Once cooled to room temperature, bags were inoculated with 7% w/w spawn in a laminar flow chamber. Finally, bags were transferred to an incubation room.

Incubation

Incubation parameters varied according to the experiment:

Experiment to assay the effects of incubation times on proximate composition and yield parameters:

Bags were spawned with strain ICFC 510/03 and incubated at 25±2 ºC in darkness during three different time lapses: 30, 50 or 70 days.

Experiment to assay the effects of strain on proximate composition and yield parameters:

Bags were spawned with strains ICFC 510/03, ICFC 879/17 and ICFC 293/00 and incubated at 25±2 ºC in darkness for 50 days.

Experiment to assay the effects of browning on proximate composition and yield parameters:

Bags were spawned with strain ICFC 510/03, and incubated at 25±2 ºC for 70 days. In order to evaluate the browning effect, 8 bags were maintained in darkness along the incubation phase while 8 were exposed to a photoperiod of 6 h of light and 18 h of darkness in the last 20 days of incubation. Light was provided with an 18 W fluorescent lamp placed on the ceiling at a distance of 2.5 m from the surface of the blocks, which generated a light intensity of 85 lux.

Cultivation room conditions and harvest

To induce fruiting, all the plastic bags were removed and blocks were carried to the cultivation room. The fruiting conditions were: temperature: 20-22 °C; humidity: 70-80%; ventilation: CO_2 less than 500 ppm; photoperiod: 9 h light and 15 h dark through a 20 W fluorescent light (400–700 nm); watering: spraying for 5 min, 5 times per day using sprinklers with a flow rate (GPM) of 0.79 - 1.14 and automatically controlled by a timer. Mushrooms were daily harvested when the cap margin was 80% open. Each mushroom was weighed and measured.

Proximate composition

Determinations of the nutritional components were carried out in the Laboratory of Food Technology and Functionality Research (LIFTA) of the Quilmes National University (Buenos Aires, Argentina) using basidiomes from the first flush. Fruiting bodies were dried at 60±2 °C to constant weight, grinded and stored in plastic bags at room temperature. Protein content was determined by the Kjeldhal method (using a conversion factor of 4.38); lipid content was estimated by the Soxhlet method. Carbohydrate content was determined as the difference between 100 and the sum of the percentages of the components mentioned above, this measure includes both simple and complex carbohydrates (fibers). Ash content was estimated according to APHA standard methods [28].

Evaluated parameters

The following production parameters were evaluated: 1) biological efficiency (BE %), calculated as the weight of fresh fruiting bodies divided by dry substrate weight x 100; 2) partial productivity (PP), calculated as the BE% divided by the number of days from induction day to the last harvest day; 3) total productivity

(TP), calculated as the BE% divided by the total cycle (days of incubation period + cropping period); 4) number of mushrooms harvested; 5) pileus diameter; 6) number of mushrooms and 7) mushroom mean weight calculated as total weight harvested during the cycle divided by the number of mushrooms obtained.

Experimental design and statistical analysis

A completely randomized design was adopted and eight replicates were used for each treatment in all experiments. Prior to analysis, the Shapiro–Wilk test and the Levene median test were applied to test data for normality and equal variation, respectively. Data were analyzed with the InfoStat software (version 2015), subjected to an analysis of variance (ANOVA) and the means were compared by the Tukey test at the 5% level.

RESULTS

Effects of incubation times on proximate composition and yield parameters

Protein, carbohydrates, fat and ash content of basidiomes yielded were determined after the inoculated bags were incubated during 30, 50 or 70 days (Fig. 1). Protein content varied from 23.5 to 23.67%, carbohydrates varied from 63.39 to 64.08%, fat content from 2.06 to 2.17% and ash content varied from 10.42 to 10.79%. No significant differences were observed in any of the components evaluated from the different incubation periods.

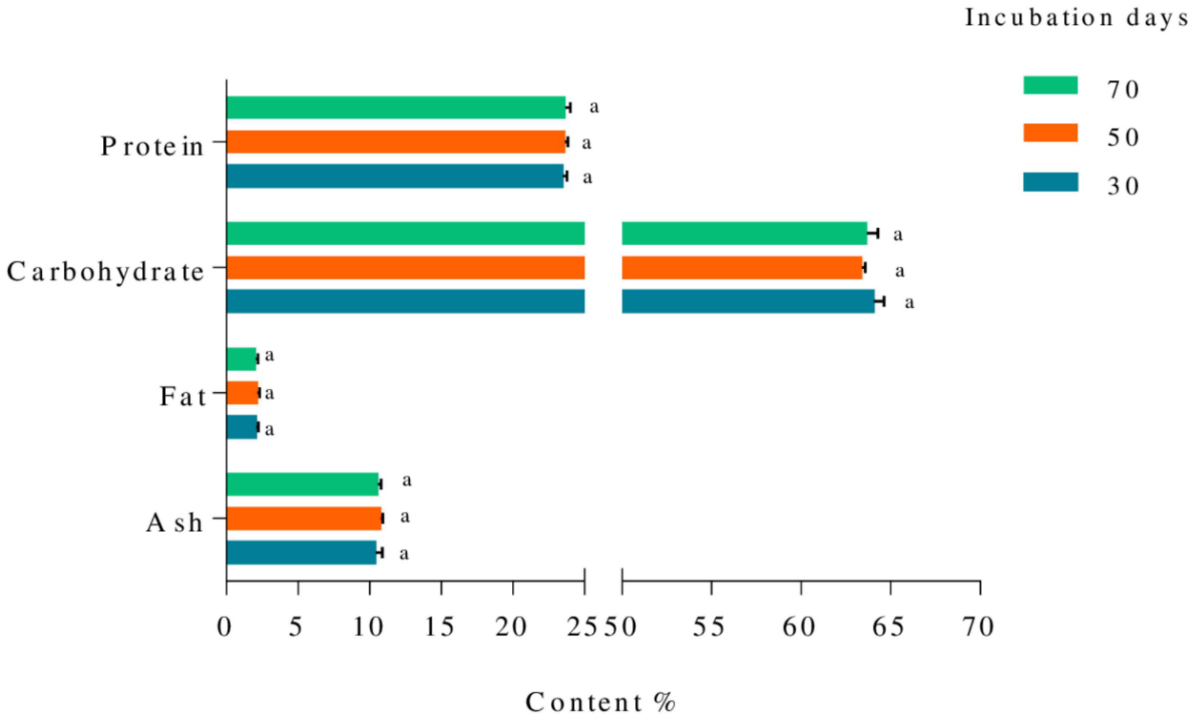

Figure 1. Protein, carbohydrate, fat, and ash content of *L. edodes* fruiting bodies (ICFC 510/03 strain) incubated during 30, 50 and 70 days. Different letters mean significant differences at $p < 0.05$, according to Tukey's test, eight replicates.

Regarding production parameters, they varied significantly according to the three incubation periods assayed (Table 1). The highest BE value was for 70 days (88.59%) followed by 50 days (72.83%) and finally for 30 days (32.00%). The highest values for 70 days of TP and PP were 0.73% per day and 1.71% per day respectively, and they were significantly different from the values of 50 days (TP: 0.53%/day and PP: 0.84%/day) and 30 days (TP: 0.46%/day and PP: 0.82%/day). The highest pileus diameter obtained was for 70 days (12.40 cm) and it was significantly different from the diameters obtained for 50 days (6.74 cm) and 30 days (5.20 cm). Regarding the highest number of mushrooms obtained it was for 50 days (32.8 units) and it was significantly different from 70 days (11.40 units) and from 30 days (8 units). Finally, the highest mushroom mean weight was for 70 days, and it was significantly different from the mushroom mean weight reached for 30 days (17.04 g) and 50 days (14.84 g).

Table 1. Biological efficiency, total and partial productivity, pileus diameter, number of mushrooms and mushroom mean weight of *L. edodes* (ICFC 510/03 strain) incubated during 30, 50 and 70 days.

Parameter	Incubation time (days)		
	30	50	70
Biological Efficiency (%)	32.00 ± 6.98[a]	72.83 ± 7.16[b]	88.59 ± 8.69[c]
Total Productivity (%/day)	0.46 ± 0.11[a]	0.53 ± 0.05[a]	0.73 ± 0.08[b]
Partial Productivity (%/day)	0.82 ± 0.20[a]	0.84 ± 0.07[a]	1.71 ± 0.22[b]
Pileus Diameter (cm)	5.20 ± 0,62[a]	6.74 ± 0.43[a]	12.40 ± 2.30[b]
Number of Mushrooms (units)	8 ± 1.00[a]	32.8 ± 6.30[b]	11.40 ± 2.30[a]
Mushroom mean weight (g)	17.04±10.04 [a]	14.84 ± 9.16[a]	49.40 ± 10,04[b]

Values in a row followed by different letters are significantly different at $p < 0.05$, according to Tukey's test, n = 8.

Effect of the use of different strains on proximate composition and yield parameters

Protein, carbohydrates, fat and ash content of basidiomes yielded were determined in three different commercial strains (Fig. 2). The proximal composition varied according to the strains analyzed. Significant differences were found among protein and ash content but not among carbohydrates or fat content. Protein content showed significant differences between strain ICFC 510/03 with 23.44% and the other two; obtaining 25.70% for ICFC 293/00 and 25.87% for strain 879/17. On the other hand, carbohydrates and fats content did not show significant differences among strains. Carbohydrates varied from 63.83 to 64.36% and fats from 1.80 to 2.13%. Ash content reached the highest value for strain ICFC 510/13 (10.39%), and it was significantly different from 293/00 and 879/17 strains (8.65% and 7.83 %, respectively).

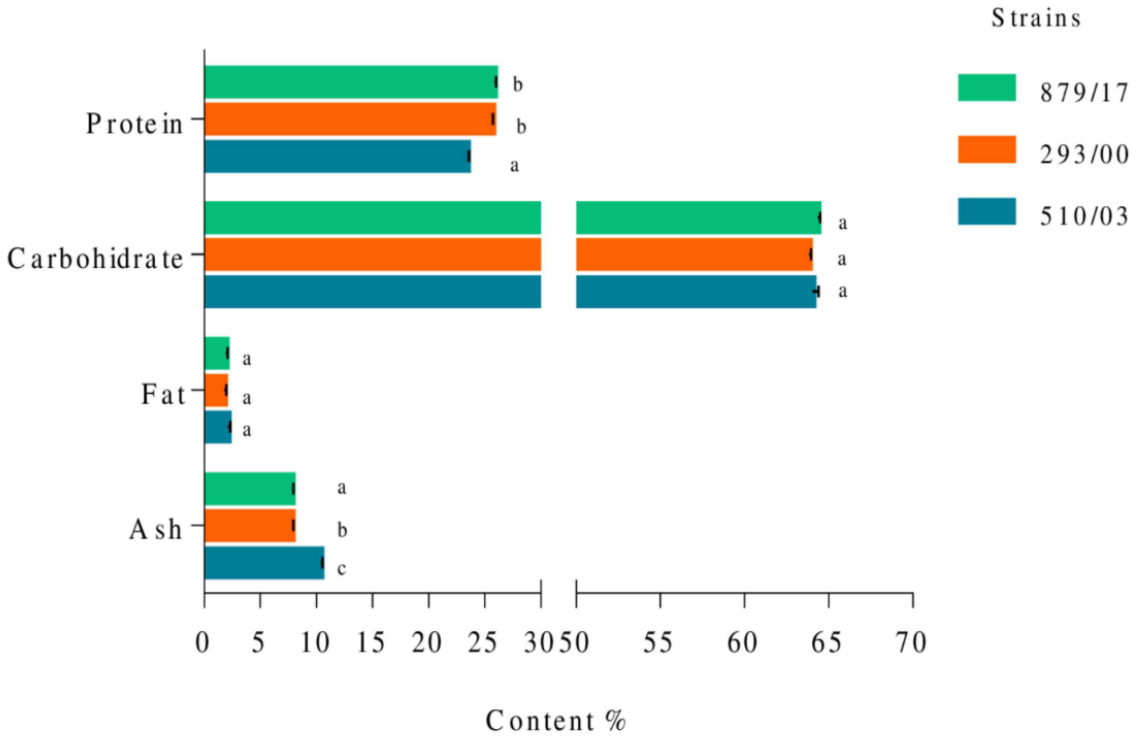

Figure 2. Protein, carbohydrate, fat, and ash content of *L. edodes* fruiting bodies of three different commercial strains. Different letters mean significant differences at $p < 0.05$, according to Tukey's test, eight replicates.

Production parameters varied significantly according to the strains assayed (Table 2). The highest BE was for ICFC 879/17 strain with 123.4% and it was significantly different from the other two strains which yielded 76.00% for ICFC 510/03 and 73.29% for ICFC 293/00. Significant differences were obtained regarding productivity among strains. The highest productivity values were obtained for ICFC 879/17 reaching 1.12%/day for TP and 2.05%/day for PP. Strain ICFC 293/00 reached 0.72%/day for TP and 1.42%/day for PP meanwhile strain ICFC 510/03 obtained the lowest values with 0.55%/day for TP and 0.86%/day for PP. Concerning the pileus diameter, significant differences were obtained among strains. The highest average was for ICFC 879/17 strain with 15.24 cm and the lower value was for strain ICFC 510/03 with 4.98 cm, while

ICFC 293/00 strain reached 7.58 cm. Concerning to the highest number of mushrooms was obtained from ICFC 510/03 strain with 38.8 units, meanwhile strains ICFC 293/00 and 879/17 produced 20.80 and 9.00 units respectively. Finally, the highest mushroom mean weight was for ICFC 879/17, and it differed significantly from the mushroom mean weight reached for ICFC 293/00 (49.29 g) and for ICFC 510/03 (31,93 g).

Table 2. Biological efficiency, total and partial productivity, pileus diameter, number of mushrooms and mushroom mean weight of three different strains of *L. edodes*.

Parameter	Strains		
	510/03	**293/00**	**879/17**
Biological Efficiency (%)	76.00 ± 8.24[a]	73.29 ± 7.78[a]	123.40 ± 2.70[b]
Total Productivity (%/day)	0.55 ± 0.09[a]	0.72 ± 0.07[b]	1.12 ± 0.03[c]
Partial Productivity (%/day)	0.86 ± 0.16[a]	1.42 ± 0.15[b]	2.05 ± 0.11[c]
Pileus Diameter (cm)	4.98 ± 0.51[a]	7.58 ± 1.04[b]	15.24 ± 2.21[c]
Number of Mushrooms (units)	38.80 ± 7.56[c]	20.80 ± 3.11[b]	9.00 ± 2.24[a]
Mushroom mean weight (g)	31,93 ± 10,73[a]	49,29 ± 15,17[a]	209 ±10,25[b]

Values in a row followed by different letters are significantly different at $p < 0.05$, according to Tukey's test, n = 8.

Effect of browning on proximal proximate composition and yield parameters

Concerning the proximal analysis, no differences were obtained when browning treatment was carried out (Fig. 3). Protein content varied from 23.44 to 23.65% meanwhile carbohydrates varied from 64.04 to 63.39%; fat content from 2.13 to 2.17% and ashes varied from 10.39 to 10.79%.

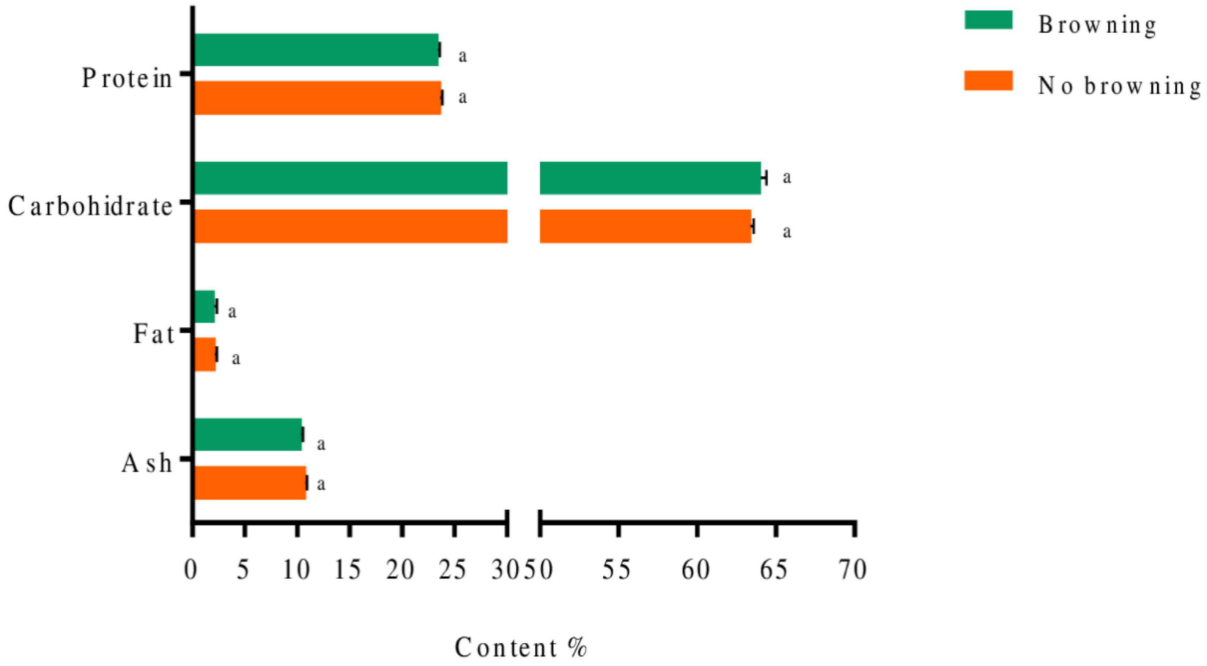

Figure 3. Protein, carbohydrate, fat, and ash content of *L. edodes* fruiting bodies with and without browning induction during incubation time. Different letters mean significant differences at $p < 0.05$, according to Tukey's test, eight replicates.

Regarding the production parameters, no significant differences were observed according to the browning effect (Table 3). BE varied from 92.41 to 88.56%; TP varied from 0.66 to 0.64 %/day and PP varied from 1.04 to 1.02 %/day; the pileus diameter varied from 4.98 to 6.74 cm and the number of mushrooms varied from 39.88 to 47.18 units. Finally, mushroom mean weight varied from 15.74 g to 12.23 g.

Table 3. Biological efficiency, total and partial productivity, pileus diameter, number of mushrooms and mushroom mean weight of *L. edodes* (ICFC 510/03 strain) with and without browning process.

Parameter	Browning	No browning
Biological Efficiency (%)	92.41 ± 8.24[a]	88.56 ± 7.16[a]
Total Productivity (%/day)	0.66 ± 0.09[a]	0.64 ± 0.05[a]
Partial Productivity (%/day)	1.04 ± 0.16[a]	1.02 ± 0.07[a]
Pileus Diameter (cm)	4.98 ± 0.51[a]	6.74 ± 0.43[a]
Number of Mushrooms (units)	47.18 ± 7.56[a]	39.88 ± 6.30[a]
Mushroom mean weight (g)	12.23 ± 10.16[a]	15.74 ± 8.24[a]

Values in a row followed by different letters are significantly different at $p < 0.05$, according to Tukey's test, n = 8.

DISCUSSION

When a mushroom farmer decides to start growing shiitake, one of the first problems they encounter is that there is a lot of information about the production cycle that is often very diverse and even contradictory. For example, the number of days required for the incubation phase vary from 3 to 10 weeks [10, 12, 16, 29, 30]. In addition to this, there is really very little information regarding the advantages of the browning process. This manuscript provides information on *L. edodes*, the most cultivated mushroom species on the planet, covering nutritional aspects and the cultivation process, in order to optimize production without losing sight of the quality of the cultivated mushrooms. Although this work aims more at considering how the proximate composition of shiitake varies in relation to the duration of incubation, the strain and browning process, it is always necessary to study the cultivation parameters to ensure that they are within the average values reported. In a first assay we evaluated three different lengths of incubation and how they influence the proximate composition and yield parameters. The results showed that there were no significant differences in the proximate composition. This means that even though the incubation time is extended, which would give the mycelia more time to digest and incorporate nutrients, it does not change its proximate composition. Regarding yield parameters, the results here obtained, have shown that the incubation time is an important variable since it affects the yield of shiitake. Incubations of 30, 50 and 70 days showed significant differences in terms of biological efficiency, productivity, pileus diameter, number of mushroom and mushroom mean weight. The highest BE% was obtained with the longest incubation time. These results agree with those published by Royse [31], who obtained higher biological efficiencies with longer spawning run times. Reasons for the increased yields when the incubation period is longer might be: greater mycelia biomass, increases in enzyme levels in the substrate and therefore the solubility of wood components [31]. Curiel Pérez and coauthors [32] however, highlighted the importance of evaluating the cultivation parameters of each strain individually, since they obtained varied responses for the optimal incubation time, 70 days for some strains and only 42 days for other strains. It is also known that the incubation time is influenced by the amount of spawn added to the substrate in many species of mushrooms [33]. When more spawn is added, incubation time, period is reduced. In this work we chose 7 % of spawn because it is the percentage commercially used in our country by mushroom farmers for shiitake cultivation [34]. Most of the shiitake farmers use 5 % of spawn for inoculation [35]. Very little information is available on how the incubation time influences the size of the harvested fruit bodies of shiitake or any other edible mushroom. Our results are in agreement with Royse [31] who reported that substrates with longer spawning run times, in general produce larger mushrooms. Thereby, incubation time should be used as a good strategy to obtain the desired size of the fruiting bodies according to the market demand or the preferences of the mushroom farmer.

In a second assay, three commercial strains of shiitake were used to evaluate the proximate composition and yield parameters. Statistical differences were found in the protein and ash content. Both ICFC 879/17 and 510/03 strains showed higher values of proteins and lower values of ashes compared to 293/00 strain. There is considerable consensus among the authors that the proximate composition of shiitake growing on a given substrate will depend on the strain that is cultivated. Gaitán-Hernández and coauthors [36] reported that the proximate composition of the *L. edodes* fruiting bodies was significantly affected by the type of strain. Protein content here obtained (from 23.44 to 25.87%) was higher than the percentage reported by Gaitán-Hernández and coauthors [36] which varied from 14.4 to 17.7% also higher than Roncero-Ramos and coauthors [37], who reported 16.82% but not so different from Dayani and coauthors [11] who reported 17.62 to 28.63%. Both carbohydrate and fat contents (from 63.83 to 64.36% and from 1.80 to 2.13%, respectively) were similar to those reported by Gaitán-Hernández and coauthors [36] (from 58.8 to 66.1% for carbohydrates and from 1.1 to 2.1% for fats) Roncero-Ramos and coauthors [37] presented similar values of

fat content (2.06%) but higher carbohydrate content (73.43 %). Ash content here obtained (from 7.83 to 10.39%) was higher than those obtained by Gaitán-Hernández and coauthors [36] (2.2 to 4.6%) but similar to the values reported by Roncero-Ramos and coauthors [37] (7.36%). Regarding the yield parameters, the strain 879/17 presented the highest productivity, the largest pileus diameter and the highest mushroom mean weight, although the lowest number of mushrooms. It is difficult to make comparisons with other works since many different substrates, strains and conditions have been studied for shiitake cultivation [20]. Comparing our results with other authors who used sawdust as the main component of the substrate and sterilization as heat treatment, the BE values that we obtained for the analyzed strains were similar or higher (73.29 to 123.40%) than the values obtained by Sharma and coauthors [38] with 81% or Manero Colín and coauthors [39] with 70.4%. Curiel Pérez and coauthors [32] obtained maximum BE values of 168%, using a substrate composed of sawdust and cotton husk and applying a cold shock to induce fruiting, thus reaching one of the highest BE values reported in the bibliography. Concerning to productivity, maximum values of total productivity here reported (1.12%/day) are slightly higher than those reached by some authors that mentioned total productivity as productivity rate (PR). Gaitán-Hernández and coauthors [36] reported PR values from 1 to 1.8%/day; Gaitán-Hernández and coauthors [40] obtained PR values from 0.6 to 1.1%/day and Royse [31] reported PR values from 0.2% to 0.7% /day. These results support the concept that strain selection for commercial cultivation is an important step to identify more productive strains [10].

In a third assay we evaluated the effects of browning on the proximate composition and yield parameters. Browning is a process induced through a photoperiod in the last phase of the incubation time which involves an extra input of energy at this point. It is a common belief that browning acts as a protective coating on the substrate, which can inhibit the invasion of pathogens and suppress water evaporation [23, 41]. Furthermore, it is a very useful indicator that the mycelium is ready for fructification [21, 29]. In this work, browning process was induced treating a group of bags with light at the final part of the incubation phase, however, we observed that at the end of the production phase, all the blocks (treated and non-treated with light) had the same appearance. That is, the browning process occurs equally in non-light treated bags in the production room although occurring later, most likely induced by photoperiod. Surprisingly, we observed that the basidiomes harvested from the non-induced blocks were of similar proximate composition to the basidiomes obtained from the blocks induced to the browning process. Furthermore, the induction of browning did not show any difference regarding BE%, productivity, pileus diameter, mushroom mean weight or number of mushrooms. No increase in the mycelia contamination was observed in the non-induced blocks. Therefore, under the controlled conditions used in this work, apparently browning is an expendable process, since it did not provide any advantage. Mushroom growers have different infrastructure and facilities in their farms, and apply different technologies to produce shiitake. Many of them perform the browning process in order to reduce contamination and loss of water from the mycelium blocks, but based on our results, tests should be carried out with each strain to corroborate if the induction of browning is really necessary. We found that there will be no contamination and no improvement in yields or changes in the proximate composition of the mushrooms with the induction of browning as long as the growing house hygiene is good and the environmental conditions are well controlled.

CONCLUSION

Based on the results we obtained, we conclude that the proximate composition of shiitake depends only on the strain and does not vary with respect to the incubation time or the induction of the browning process. Having also studied yield parameters such as BE%, productivity, number of produced mushrooms and diameter of fruiting bodies, we verified that the incubation time has affected these parameters. In this way, longer periods of incubation produced higher yields with increasing BE and productivity. Although it is believed that blocks of shiitake that have been induced to browning are better prepared for the production phase, this process, its application, costs and management should be evaluated by mushroom growers considering that the induction of browning will not improve yields neither the proximate composition of the produced mushrooms.

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Conflicts of Interest: The authors declare no conflict of interest.

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