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Biovalorization of Grape Stalks as Animal Feed by Solid State Fermentation Using White-Rot Fungi

Valéria Costa-Silva [1], Mariana Anunciação [1], Ederson Andrade [2], Lisete Fernandes [3], Aida Costa [4], Irene Fraga [5], Ana Barros [5], Guilhermina Marques [5], Luís Ferreira [5] and Miguel Rodrigues [5,*]

1 Animal and Veterinary Research Centre (CECAV), Department of Animal Science, University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal; valeriasilva@utad.pt (V.C.-S.); marianaanunciacao@hotmail.com (M.A.)
2 Department of Animal Science, Federal University of Bahia, Salvador 40170110, Brazil; edersonzootec@gmai.com
3 Chemistry Department (ECVA), University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal; lisfernandes@utad.pt
4 Floresta Viva, Golas Street, 466-748 Amarante, Portugal; info@florestaviva.pt
5 Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal; ifraga@utad.pt (I.F.); abarros@utad.pt (A.B.); gmarques@utad.pt (G.M.); lmf@utad.pt (L.F.)
* Correspondence: mrodrigu@utad.pt

Abstract: This work aimed to evaluate the potential of three fungi strains, *Lentinula edodes*, *Pleurotus eryngii*, and *Pleurotus citrinopileatus*, to degrade lignin and enhance the nutritive value of grape stalks (GS). The GS was inoculated with the fungi and incubated under solid-state fermentation at 28 °C and 85% relative humidity for 7, 14, 21, 28, 35, and 42 days, in an incubation chamber. The influence of the treatments was evaluated by analyzing the potential modifications in the chemical composition, *in vitro* organic matter digestibility (IVOMD) and enzymatic kinetics. An increase ($p < 0.001$) in the crude protein content was observed in the GS treated with *L. edodes* and *P. citrinopileatus* at 42 days of incubation (50 and 75%, respectively). The treatment performed with *L. edodes* decreased ($p < 0.001$) lignin content by 52%, and led to higher ($p < 0.001$) IVOMD values at 42 days of incubation. By contrast, *P. eryngii* did not affect lignin content and IVOMD. A higher activity of all enzymes was also detected for the treatment with *L. edodes*. Results indicated that *L. edodes* has a great potential to enhance the nutritive value of GS as an animal feed, due to its lignin degradation selectivity.

Keywords: wine by-products; grape stalks; solid state fermentation; white-rot fungi; *in vitro* digestibility; enzymatic activities

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1. Introduction

The wine industry is one of the most important agricultural activities throughout the world and produces large amounts of agro-industrial residues, representing a waste management concern, both ecological and economically [1,2]. Grape stalks (GS) are one of those by-products, obtained by the destalking procedure just before the wine fermentation process. On average, 4 kg of GS are generated per hectoliter of produced wine [3,4]. Recently, there is a growing interest in reducing the impact of agro-industrial wastes by using them as possible raw materials for new feed products and other applications, thus enhancing the contribution to the general policy framework inherent to the implementation of a circular economy [2,5,6].

Similarly, to other lignocellulosic biomass, GS presents a low protein content (<30 g/kg DM) and a high (>50 g/kg DM) lignified cell wall content [7,8] that limits feed intake and animal performance [9,10]. Lignin is a phenolic compound with a high molecular weight that adds rigidity to the cell wall structure, being resistant to most microbial enzymatic systems.

Consequently, the digestibility and availability of cell wall contents to rumen microorganisms are low [11,12]. Among other factors, a low access of rumen microbial enzymes to structural carbohydrates is due to the linkages between lignin and cellulose and hemicelluloses, hindering the availability of significant amounts of potential energy [7,12,13] that could otherwise be used by the microbial population in the rumen. Thus, the utilization of methodologies to break down lignin–carbohydrate linkages is essential to improve the access of rumen microbes to cellulose and hemicellulose, and consequently enhance their digestibility.

Different treatments of lignocellulosic biomass have been studied for improving its nutritive value. In general, physical, and chemical treatments are not cost-effective and are not environmentally friendly processes. By contrast, biological treatments have been drawing more attention due to their low costs, simplicity, higher yields, and, simultaneously, their higher sustainability. Additionally, the conversion of lignocellulosic biomass into more nutritive and easily digestible products has been underlined by several authors [14–16].

Solid state fermentation (SSF) is one of the possible biological treatments that is known to be able to reproduce the natural culture conditions of microorganisms; it can cope with substantial volumes of biomass and have a low environmental impact and reduced water consumption [17]. Many microorganisms are capable of degrading lignin by using cellulose and hemicellulose as carbon and energy sources [18]. A small group of filamentous fungi, known as white-rot fungi (WRF), has evolved in different ecological frameworks, enhancing their selective lignin-degrading ability due to their rich network of ligninolytic enzymes [18,19]. WRF can maximize the production of lignocellulolytic enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. These enzymes oxidize lignin and a wide range of lignin analogous compounds and break down lignin–carbohydrate linkages [20].

The use of WRF as a biological treatment has been widely reported [7,21–23] as a promising, affordable, and environmentally acceptable approach, where an increase of the nutritive value of several raw materials was observed. However, changes in lignin degradation depend not only on the chemical composition of those raw materials, but also on the fungus species and incubation period used [7,24]. In this context, the purpose of this study was to evaluate the effect of incubation periods (28, 35, and 42 days) and fungal species (*Lentinula edodes*, *Pleurotus citrinopileatus* and *Pleurotus eryngii*) on the improvement of GS nutritive value, analyzing the effects on its chemical composition and *in vitro* organic matter digestibility.

2. Materials and Methods

2.1. Fungal Cultures and Spawn Preparation

Three WRF strains, *Lentinula edodes* (UF21403), *Pleurotus eryngii* (UF21402), and *Pleurotus citrinopileatus* (UF21401) were inoculated on potato dextrose agar (PDA) plates and incubated at 28 °C until the mycelia covered the entire surface. For inoculum spawn preparation, wheat grains were previously soaked in distilled water for 12 h to ensure their moisture content for fungal growth. The excess water was discarded, and the grains were autoclaved in bags with filters (filter type T; filter pore size of 0.2 µm; Unicornbags, Plano, TX, USA), for 15 min at 121 °C, to prevent any contamination. After the sterilization period, bags were left to cool until room temperature. At this point, a sample with approximately 3 cm^2 from each inoculated plate with the different WRF was placed in several bags. The bags were kept at 28 °C for 20 days until almost all grains were colonized by the mycelia. The schematic presentation for the preparation of inoculum spawn is represented in Figure 1a–d.

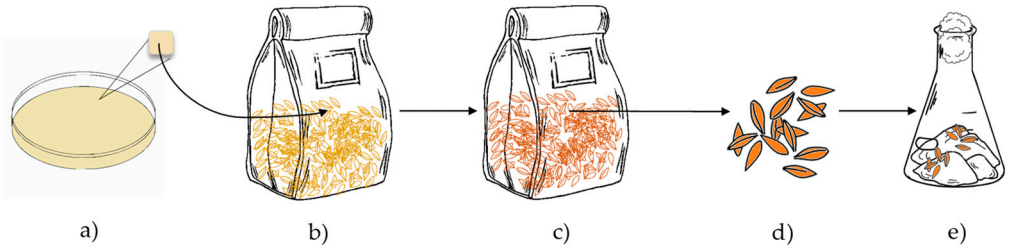

Figure 1. Schematic presentation of the preparation of inoculum spawn (**a**–**d**) and the experiment of solid state fermentation with WRF on GS (**e**); (**a**) Plates with the entire surface covered by mycelium; (**b**) Incubation bag with filter with sterilized wheat grain; (**c**) Inoculum spawn (after 20 days approximately); (**d**) Wheat grain with mycelium; (**e**) Grape stalks with inoculum spawn.

2.2. Substrate Preparation and Biological Pre-Treatment

GS samples were collected in wine cellars after the grape destemming process from the *Região Demarcada do Douro*, in northern Portugal. All samples were air-dried at 60 °C, milled to a 3 mm screen (Ultra Centrifugal Mill Type ZM200, Retsch, Haan, Germany), and hermetically stored at room temperature. Before fungal inoculation, the substrate was previously soaked in distilled water for 12 h, and afterward, the excess water was removed. Incubation was conducted in 500 mL Erlenmeyer flasks containing 50 g of the humidified substrate and closed with a cotton plug. These flasks were autoclaved at 121 °C for 30 min, cooled, and inoculated with approximately 2 g of each inoculum spawn. The schematic presentation of the experiment of solid state fermentation with WRF of GS is represented in Figure 1e. The inoculated substrate was incubated in quadruplicate along with a control (autoclaved substrate with 2 g of non-inoculated wheat grain) at 28 °C and 85% relative humidity, during three distinct incubation periods (IP; 28, 35, and 42 days). At the end of the different incubation periods, crude enzyme extracts were prepared by adding 120 mL of deionized water followed by an incubation on a rotary shaker at 100 rpm for 3 h. After solid/liquid separation, chilled extracts were filtered (Whatman GF/A), centrifuged, and aliquots were stored at −20 °C before the determination of enzyme activities.

2.3. Chemical Analyses

To determine dry matter (DM) content, samples were dried to constant weight in an air-forced oven at 40 °C and milled with a 1 mm screen (Tecator Cyclotec 1093 Sample Mill, FOSS, Hillerød, Denmark). Neutral detergent fiber (NDFom), acid detergent fiber (ADFom), and lignin(sa) (determined by the solubilization of cellulose with sulfuric acid and expressed exclusive of residual ash) fractions were determined without the use of sodium sulfite and α-amylase, based on the methods proposed by Van Soest et al. [25]. The concentration of hemicellulose was calculated as the difference between NDFom and ADFom, and that of cellulose as the difference between ADFom and lignin(sa). Dried samples were analyzed for ash (no. 942.05) in porcelain crucibles, which were placed in a temperature-controlled furnace (Vulcan, A-550, Torrance, CA, USA) preheated to 550 °C, and total N as Kjeldahl N (no. 954.01) was measured, in which N obtained from the digested samples with sulfuric acid was calculated after a distillation and titration procedure according to AOAC methods [26]. The crude protein (CP) content was calculated as N × 6.25.

2.4. In Vitro Organic Matter Digestibility

In vitro organic matter digestibility (IVOMD) was calculated following the procedures of Tilley and Terry [27], modified by Marten and Barnes [28]. Rumen fluid was collected from two non-lactating Holstein–Friesian cows fed a diet consisting of corn silage (70%), concentrate feed (25%), and hay (5%), given twice a day (morning and afternoon) in equal amounts. The rumen fluid was collected 2 h after the morning meal in a preheated and CO_2-

saturated container, which was previously filtered through cheesecloth. All manipulations were performed under the continuous flow of CO_2.

2.5. Enzymatic Activities

2.5.1. Carboxymethylcellulase

To determine the carboxymethylcellulase activity, a 1.0% (*w*/*v*) substrate solution of carboxymethyl cellulose (CMC) (Sigma C-4888) in 50 mM citrate buffer pH 4.8 was previously prepared. Briefly, 250 µL of crude enzyme extract and 500 µL of substrate (CMC) were added to each test tube. Subsequently, the test tubes were placed in a shaking incubator (Gesellschaft für Labortechnik—3032, Burgwedel, Germany) with an orbital shaker at 100 rpm, for 30 min at 45 °C [29]. The hydrolyzed reducing sugars were determined by the dinitrosalicylic acid (DNS) method, according to the procedure proposed by Miller [30]. After the incubation period, 3 mL of DNS was added to stop the reaction and chromophore development. A calibration curve of 100 to 1500 µg mL^{-1} glucose was prepared, and measurements were performed at 540 nm in a Helios gamma UV-vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA).

2.5.2. Avicelase

A 1.0% (*w*/*v*) avicel substrate solution in citrate buffer (pH 4.8 to 50 mM) was prepared. Briefly, 250 µL of crude enzyme extract and 500 µL of substrate (Avicel) were added to each test tube. Subsequently, the test tubes were placed in a GFL incubator with an orbital shaker at 45 °C at 100 rpm for 180 min [29]. After this period, 3 mL of DNS was added and the quantification of the reducing sugars was performed as described in 2.5.1.

2.5.3. Xylanase

A substrate solution of 1% (*w*/*v*) xylan (birchwood xylan) in 0.1 M potassium phosphate buffer at pH 6.0 was prepared. The mixture was incubated for 60 min at 45 °C and 100 rpm [31]. After this period, the quantification of the reducing sugars was performed according to the two previous enzymes, using a calibration curve from 100 to 1500 µg mL^{-1} of xylose.

2.5.4. Feruloyl Esterase

The assay of feruloyl esterases was performed as reported by Mastihuba et al. [32] and Dinis et al. [33] through a spectrophotometric method by measuring the production of 4-nitrophenol (4NP) from 4-nitrophenyl ferulate (4NPF). Briefly, the activity of feruloyl esterase was calculated through the addition of 100 µL of enzyme extract and 2000 µL of substrate in 100 mM of potassium phosphate-buffered solution at pH 6.5 in a test tube. The mixture was placed in a static incubator for 30 min at 50 °C in the dark. The 4NP released from the 1 mM substrate solution (final concentration) was determined by the absorbance at 410 nm in a Helios gamma UV-vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Absorbance was converted into concentration through a standard curve prepared with 4NP (0.05 to 0.5 mM).

2.5.5. Laccase

Laccase activity was measured following the oxidation of 2,2-azino-bis (3-ethylbenzth iazoline-6-sulfonic acid (ABTS) (ε 420 = 36 mM^{-1} cm^{-1}) in a Helios gamma UV-vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). The assay was performed at 25 °C and the mixture contained 1200 µL of 100 mM phosphate–citrate buffer at pH 4.0, as well as 200 µL ABTS 30 mM and 100 µL of crude enzyme extract [29].

2.5.6. Manganese Peroxidase

Manganese peroxidase activity was determined by the production of a Mn^{3+}–tartrate complex (ε 238 = 6.5 mM^{-1} cm^{-1}) in a Helios gamma UV-vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) from 1.5 mM using 100 mM sodium tartrate buffer

at a pH of 5.0 and 10 mM H_2O_2. Briefly, the activity of MnP was determined through the addition of 1185 µL of sodium tartrate buffer, 200 µL of $MnSO_4$, 100 µL of crude enzyme extract, and 15 µL of H_2O_2 [30].

2.5.7. Lignin Peroxidase

Lignin peroxidase activity was measured by the oxidation of 2 mM veratraldehyde alcohol (ε 310 = 9.3 mM^{-1} cm^{-1}) in a Helios gamma UV-vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) from 100 mM sodium tartrate buffer at a pH of 3 in the presence of 10 mM H_2O_2. Lignin peroxidase activity was performed under the same conditions as manganese peroxidase [30], and was expressed in enzyme units, defined as l µmol of substrate oxidized per minute, under the assay conditions.

2.6. Scanning Electron Microscopy

Scanning electron microscopy in environmental mode (SEM/ESEM FEI QUANTA-400, Hillsboro, OH, USA) was performed after the preparation of the different samples of GS, after being fixed with carbon double-sided tape on aluminum stubs. For the visualization we used Low Vacuum Mode, with a partial pressure inside the chamber of 1.30 mbar and an acceleration voltage between 10 and 20 kV. Several images were acquired with different magnifications.

2.7. Statistical Analysis

Statistical analysis was carried out using JMP program version 14 (SAS, 2018). The effects of WRF treatment (*L. edodes, P. citrinopileatus,* and *P. eryngii*) and incubation period (28, 35, and 42 days) and their interactions with the chemical composition and IVOMD of the GS were examined by an analysis of variance (ANOVA). The effects of WRF treatment and incubation period (7, 14, 21, 28, 35, and 42 days) and their interaction with the enzymatic activities (carboxymethylcellulase, avicelase, xylanase, feruloyl esterase, laccase, manganese peroxidase and lignin peroxidase) were examined by an analysis of variance (ANOVA). Differences were considered statistically significant at $p \leq 0.05$. Principal components analysis (PCA) was also used to explore the differences between WRF treatment in chemical composition, IVOMD, and enzymatic activities.

3. Results and Discussion

The chemical composition and *in vitro* organic matter digestibility (IVOMD) of the treated and untreated (control) grape stalks (GS) are presented in Table 1. Results showed an effect ($p < 0.001$) of the white-rot fungi (WRF) treatment on all chemical parameters. Data indicated a decrease of the DM content of GS treated with the WRF when compared with the control ($p < 0.05$), except for *P. eryngii* at 35 and 42 days, probably due to the observed decrease of activity of the mycelium on the substrate for these periods.

Regarding ash content, an increase was observed for all GS treated with the WRF when compared with the control ($p < 0.05$). Ash content for *P. eryngii, L. edodes* and *P. citrinopileatus* showed an average increase of 22%, 47%, and 68%, respectively. This increase in ash was also reported by other authors when inoculating different substrates with WRF [34,35].

The data indicated a higher CP content of GS treated with the WRF when compared with the control ($p < 0.05$), showing increases of 32, 78, and 106% for *P. eryngii, L. edodes,* and *P. citrinopileatus*, and at 42 days of incubation, respectively. These data are consistent with results presented in other studies [16,25,36–38]. One possible explanation for this increase could be related to the analytical method used for N quantification. The Kjeldahl N method does not quantify all forms of inorganic nitrogen (e.g., nitrates and nitrites; [38]) and fungi are known to possess nitrate and nitrite reductases, allowing them to convert these inorganic forms of nitrogen into quantifiable Kjeldahl N method-measurable ammonia [39], thus contributing to an increase in the total N measurements after incubation. Data showed that the WRF treatment promoted changes ($p < 0.001$) in the GS cell wall components. A decrease in NDFom and ADFom contents was observed on treated GS with *L. edodes* when compared

with the control ($p < 0.05$), with a decrease of 14 and 20% at 42 days of the incubation period, respectively. Similar results were also reported in other studies [22,35,40–42] when incubating *L. edodes* with other raw materials. In fact, Tuyen et al. [22] found a decrease of 30 and 18% in NDF and ADF contents, respectively, after 49 days of incubation of wheat straw with *L. edodes*. Rahman et al., [40] also observed a decrease of 15 and 20% in NDF and ADF content, respectively, after 63 days of incubation of oil palm fronds treated with *L. edodes*. In contrast, when treating GS with *P. eryngii*, an increase in the NDFom content was observed, with an average 6% when compared to the control.

Table 1. Chemical composition (g/kg DM) and IVOMD (g/kg OM) of control and GS after 28, 35, and 42 days of incubation period with three different WRF.

IP	Fungi	DM	Ash	CP	NDFom	ADFom	HC	Cel	Lignin(sa)	HC/L	Cel/L	IVOMD
	Control	31.5 ^{a}	5.6 ^{f}	4.8 ^{d}	68.3 ^{cd}	62.1 ^{abc}	7.02 ^{cd}	27.9 ^{c}	33.7 ^{ab}	0.21 ^{ef}	0.84 ^{c}	289 ^{d}
28	*L. edodes*	22.4 ^{ef}	8.3 ^{c}	8.2 ^{b}	63.0 ^{e}	53.1 ^{de}	9.9 ^{a}	30.6 ^{abc}	22.7 ^{e}	0.44 ^{bc}	1.36 ^{b}	380 ^{c}
	P. citrinopileatus	21.1 ^{f}	9.1 ^{b}	8.8 ^{b}	70.1 ^{bc}	61.2 ^{bc}	10.34 ^{a}	30.7 ^{abc}	28.1 ^{cd}	0.37 ^{cd}	1.09 ^{c}	287 ^{d}
	P. eryngii	26.2 ^{bcd}	6.8 ^{de}	6.6 ^{c}	73.0 ^{a}	65.3 ^{ab}	7.9 ^{bcd}	31.4 ^{abc}	34.2 ^{a}	0.23 ^{ef}	0.93 ^{c}	268 ^{d}
35	*L. edodes*	25.1 ^{cde}	8.0 ^{d}	8.4 ^{b}	60.6 ^{ef}	51.7 ^{e}	8.9 ^{abc}	32.6 ^{a}	18.7 ^{f}	0.48 ^{b}	1.73 ^{a}	448 ^{b}
	P. citrinopileatus	21.3 ^{f}	9.5 ^{ab}	9.0 ^{ab}	68.7 ^{cd}	58.9 ^{c}	9.6 ^{a}	29.4 ^{bc}	29.0 ^{bcd}	0.33 ^{de}	1.01 ^{c}	289 ^{d}
	P. eryngii	28.6 ^{ab}	7.1 ^{d}	6.7 ^{c}	72.1 ^{ab}	66.8 ^{a}	6.8 ^{d}	33.0 ^{a}	33.6 ^{a}	0.20 ^{f}	0.98 ^{c}	292 ^{d}
42	*L. edodes*	23.4 ^{def}	8.4 ^{c}	8.5 ^{b}	58.7 ^{f}	49.1 ^{e}	9.6 ^{a}	32.0 ^{ab}	16.1 ^{f}	0.60 ^{a}	2.00 ^{a}	496 ^{a}
	P. citrinopileatus	20.8 ^{f}	9.8 ^{a}	9.9 ^{a}	66.3 ^{d}	56.9 ^{cd}	9.4 ^{ab}	28.6 ^{def}	27.2 ^{d}	0.35 ^{cd}	1.06 ^{c}	344 ^{c}
	P. eryngii	28.0 ^{abc}	6.5 ^{e}	6.3 ^{c}	72.2 ^{ab}	65.5 ^{ab}	7.4 ^{cd}	32.6 ^{a}	31.9 ^{abc}	0.23 ^{ef}	0.99 ^{c}	293 ^{d}
	SEM	0.96	0.27	0.30	0.78	1.33	0.46	0.81	1.10	0.017	0.081	1.13
	Effects											
	IP	0.061	0.764	0.250	<0.001	0.030	0.032	0.439	0.001	0.031	0.003	<0.001
	Fungi	<0.0001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	IP × Fungi	0.391	0.069	0.051	0.017	0.157	0.638	0.037	0.009	0.003	<0.001	<0.001

Values with different letters within a column are significantly ($p < 0.05$) different. IP, incubation period (day); DM, dry matter; OM, organic matter; CP, crude protein; NDFom, neutral detergent fiber; ADFom, acid detergent fiber; HC, hemicellulose; Cel, Cellulose; IVOMD, *in vitro* organic matter digestibility.

These results could be linked to the low biodegradation of cell wall components performed by *P. eryngii* on GS when compared with the other used fungi. Tuyen et al. [42] studied the effects of different WRF (*Ceriporiopsis subvermispora*, *L. edodes*, *P. eryngii*, and *P. ostreatus*) on different raw materials (maize stover, rice straw, oil palm fronds, and sugarcane bagasse) with an incubation time of 42 days and they reported lowest losses in the cell wall contents of the raw materials when using *P. eryngii*. In addition, it should also be stated that another reason for this increase might also be due to the proportionally higher hydrolysis potential of soluble components when compared to the degradation of cell wall components.

The modifications observed in the NDFom and ADFom fractions can be the result of the WRF activity on the three-dimensional structure of the cell wall, enhancing both the degradation of structural carbohydrates (hemicellulose and cellulose) and the solubilization of lignin. The results herein presented for the cell wall components were mainly due to the delignification of the cell wall instigated by the WRF. In fact, the incubation of GS with *P. citrinopileatus* and *L. edodes* resulted in a decrease of −17 and −43% of lignin content, respectively. In the case of *L. edodes*, the delignification process was positively related to the incubation period, decreasing lignin content by −33, −45 and −52% after 28, 35 and 42 days of incubation, respectively. Based on these results it is possible that a longer incubation period could enhance a higher delignification of cell wall contents, since the values have not reached a plateau. Similar results in delignification were found by Rahman et al. [40] when inoculating oil palm fronds with *L. edodes*. In fact, it was observed that there was a decrease in the lignin content of this substrate by −30 and −58% after 21 and 63 days of incubation, respectively. By contrast, the treatment with *P. eryngii* only showed a decrease of 5% of the lignin content of GS, even after 42 days of incubation. Okano et al. [43] also observed a small delignification (−15%) ability of *P. eryngii* on sugarcane bagasse after a longer incubation period (155 days).

According to Zheng et al. [16], the ratio of structural carbohydrates/lignin is an important measure to evaluate the selectivity of fungi to degrade lignin and, at the same time, retain the structural carbohydrates. In non-selective fungi, delignification is associated with important structural carbohydrates losses. These structural carbohydrate losses represent a disadvantage when the objective is the valorization of different substrates for animal production, since these structural carbohydrates could be used as an energy source by the animals. During the incubation period of GS, the ratio of hemicellulose/lignin (Table 1) increased from 0.21 (control) to an average of 0.35 (*P. citrinopileatus*) and 0.60 (*L. edodes*) at 42 days of incubation. For the ratio of cellulose/lignin, an increase ($p < 0.001$) in its value was only observed in the GS treated with *L. edodes*, increasing from 0.84 (control) to 2.00 at the same incubation time (Table 1). These results (Figure 2) indicated that *L. edodes* showed a higher selectivity for the delignification of GS when compared to the consumption of the structural carbohydrates.

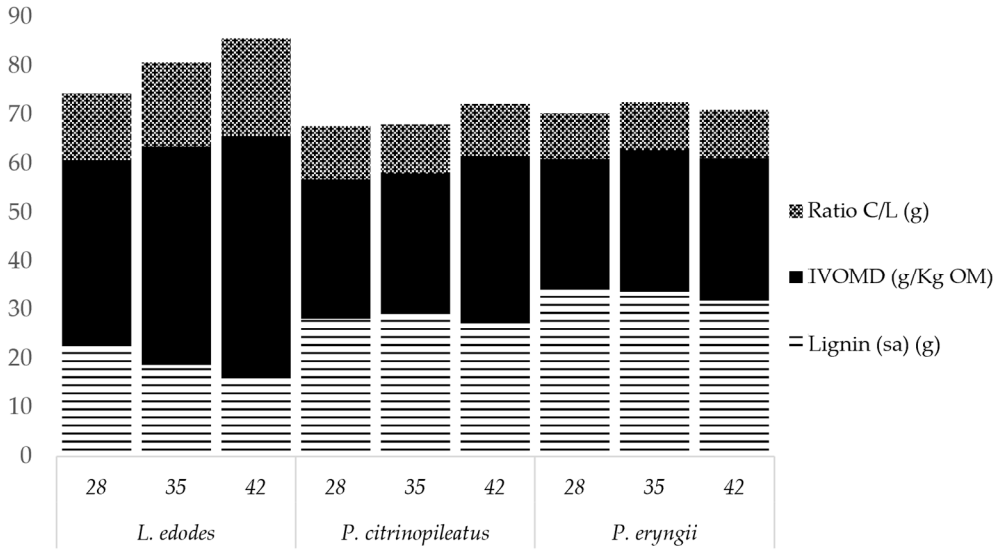

Figure 2. The ratio of cellulose (C) to lignin (sa) (L) in the dry matter, IVOMD, and lignin of GS treated with different fungi for 28, 35, and 42 days.

In fact, other authors [40,44] reported that *L. edodes* is a potential fungal species for the treatment of different raw materials with high lignin content. Rahman et al. [40] studied the effects of the incubation of ten WRFs on oil palm fronds and determined the relative selectivity for lignin degradation according to the equation proposed by Fackler et al. [45]. After comparing the results between the WRF, these authors observed that *L. edodes* presented a higher relative selectivity for lignin degradation (Srel = 0.98). Van Erven et al. [44] compared the selective delignification of wheat straw by three WRF (*C. subvermispora*, *P. eryngii*, and *L. edodes*) across the quantification of initial and final lignin content. These authors observed that *C. subvermispora* and *L. edodes* outperformed *P. eryngii* in terms of the extent and selectivity of delignification.

As a consequence of the abovementioned modifications in the cell wall contents, IVOMD was altered ($p < 0.001$) by the fungal treatments. The data clearly showed differences in the fungal degradation activity, indicating higher IVOMD values of GS treated with *L. edodes* (496 g/kg OM) and *P. citrinopileatus* (344 g/kg OM) when compared to the control (289 g/kg OM), with *L. edodes* being the most effective fungi-enhancing IVOMD. In fact, for *L. edodes*, a constant increase of IVOMD was observed across all incubation periods, varying between 32, 55, and 72% for incubation times of 28, 35, and 42 days, respectively. As in the delignification process, IVOMD was positively associated with the increase of the incubation period (Figure 2). By contrast, incubation with *P. eryngii* did not affect the IVOMD of GS. Similar results were observed by other authors [24,40,46,47], when evaluating the effects on digestibility after incubating different raw materials with distinct fungal

strains. Van Kuijk et al. [48] observed an increase in the *in vitro* gas production of wheat straw and oak wood chips, after being incubated with *L. edodes* for 56 days. Tuyen et al. [42] also observed an increase of *in vitro* rumen fermentation after the incubation of four raw materials (maize stover, rice straw, oil palm fronds, and sugarcane bagasse) with four WRF. In our study, samples treated with *L. edodes* increased the IVOMD throughout the fungal incubation period (0 to 42 days). By contrast, no effect of the fungal incubation period was observed for *P. eryngii*. These results support the low effect of *P. eryngii* treatment in improving IVOMD, and this could be due to the irrelevant effect of this fungus on the delignification of GS (Table 1).

IVOMD differences among the studied treatments can be explained by specific modifications in the cell wall structure instigated by distinct WRFs during the incubation period (Figure 2). Low digestibility values in raw materials could be linked to the presence of lignin. The linkages between lignin and cellulose and hemicellulose interfere in the access of rumen microbial enzymes to the structural carbohydrate present in cell walls [7]. The fungal enzymatic system, especially that of WRF, breaks down those lignin linkages and simultaneously converts polysaccharides into simple monomer components [49]. Thus, lignin degradation enhances digestibility through the release of the more accessible cellulose and hemicellulose, which can be easily digested by rumen microbiota [20,50]. In fact, WRF enzyme activities are characterized by the production of several extracellular enzymes that directly degrade the lignin, cellulose, and hemicellulose of the plant cell wall. These enzymatic complexes seem to exert their effect on the cell wall structure due to a concerted action between the different types of enzymes [33], and this may promote different types of synergetic efficiency in the degradation processes. According to Anand et al. [50] both the physiological conditions for lignin degradation and the expressed enzyme systems differ between fungal species. Differences may be linked to the taxonomic position of the fungi, their substrate specialization, and stage of degradation, among other characteristics [22,51]. In this way, there has been a growing research interest to characterize and quantify these ligninolytic enzymes for their ability to solubilize the lignocellulosic complex [18,52].

The enzymatic activities of seven enzymes: laccase (Lacc), manganese peroxidase (MnP), lignin peroxidase (LnP), carboxymethyl cellulase (CMCase), avicelase, xylanase, and feruloyl esterases (FAEs), were evaluated (Table 2) for the incubation of three different strains of fungi (*L. edodes, P. citrinopileatus*, and *P. eryngii*) in six different incubation periods (IP; 7, 14, 21, 28, 35 and 42 days). These enzymes can be divided into three important classes: enzymes involved in ligninolysis, cellulolysis, and hemicellulolysis [50]. Regarding the enzymes involved in ligninolysis, there are three main oxidative enzymes secreted by WRF: peroxidases (LiP and MnP) and Lacc, responsible for decomposing a variety of refractory compounds ranging from simple phenolic molecules to xenobiotics [50,52]. All fungi had an increasing production of Lacc, achieving the peak of production until 35 days of IP, with *L. edodes* showing the highest production (3.62 U g^{-1} DM; $p < 0.001$). The same trend was observed for MnP production kinetics with the exception of *L. edodes*, which maintained the peak until 42 days of incubation (2.77 U g^{-1} DM; $p < 0.001$). Higher LnP activities were also measured for longer incubation periods, with *L. edodes* presenting the highest production at 42 days of incubation (2.33 U g^{-1} DM; $p < 0.001$). It should be noticed that no LnP activity was detected for *P. eryngii* in any of the IPs. Studies have shown that for several white-rot fungi strains, activities of MnP and laccase are predominant [33] and that LnP activities are not always detected [53].

Nevertheless, one must refer that the efficient segregation of lignocellulolytic enzymes during the SSF fungal treatment of agro-industrial wastes is quite variable and it depends on several factors such as the strain specificity, duration of incubation periods and incubation conditions. The effects of the interaction between the IP and the fungi clearly denote these differences, enhancing the differential behavior of the different fungi according to the IP.

Table 2. Enzymatic activities (U g^{-1} DM) were measured at 6 different incubation periods (7, 14, 21, 28, 35, and 42 days) of GS in solid state fermentation with *L. edodes*, *P. citrinopileatus*, and *P. eryngii*.

IP	Fungi	Lacc	MnP	LnP	Avicelase	CMCase	Xylanase	FAEs
7	*L. edodes*	0.114 ^{jk}	0.339 ^{j}	0 ^{i}	0.200 ^{fg}	0.409 ^{f}	0.747 ^{g}	0.144 ^{j}
	P. citrinopileatus	0.279 ^{j}	0.087 ^{k}	0 ^{i}	0.159 ^{ij}	0.400 ^{fg}	0.463 ^{i}	0.879 ^{ef}
	P. eryngii	0 ^{k}	0.120 ^{k}	0 ^{i}	0.092 ^{l}	0.179 ^{ij}	0.294 ^{j}	0.802 ^{h}
14	*L. edodes*	0.670 ^{i}	1.896 ^{e}	0.259 ^{h}	0.580 ^{a}	1.509 ^{a}	1.0 ^{f}	0.379 ^{i}
	P. citrinopileatus	1.451 ^{g}	1.624 ^{f}	0.266 ^{h}	0.233 ^{de}	0.600 ^{cd}	0.637 ^{gh}	1.138 ^{c}
	P. eryngii	0.858 ^{h}	0.383 ^{j}	0 ^{i}	0.356 ^{c}	0.456 ^{ef}	1.148 ^{f}	0.839 ^{fgh}
21	*L. edodes*	1.581 ^{fg}	2.355 ^{cd}	1.490 ^{d}	0.221 ^{ef}	0.620 ^{c}	1.577 ^{d}	0.820 ^{gh}
	P. citrinopileatus	1.714 ^{ef}	1.770 ^{ef}	0.494 ^{g}	0.253 ^{d}	0.654 ^{c}	0.681 ^{gh}	1.151 ^{c}
	P. eryngii	1.641 ^{f}	0.738 ^{i}	0 ^{i}	0.174 ^{hi}	0.256 ^{hi}	0.541 ^{hi}	0.855 ^{fg}
28	*L. edodes*	2.734 ^{b}	2.539 ^{bc}	1.820 ^{c}	0.203 ^{fg}	0.471 ^{ef}	2.034 ^{c}	1.016 ^{d}
	P. citrinopileatus	2.093 ^{c}	1.895 ^{e}	1.119 ^{d}	0.401 ^{b}	0.938 ^{b}	1.318 ^{e}	1.218 ^{b}
	P. eryngii	1.856 ^{de}	0.945 ^{h}	0 ^{i}	0.152 ^{ij}	0.212 ^{ij}	0.472 ^{i}	0.880 ^{ef}
35	*L. edodes*	3.618 ^{a}	2.598 ^{ab}	1.995 ^{b}	0.181 ^{gh}	0.426 ^{f}	2.348 ^{b}	1.168 ^{c}
	P. citrinopileatus	2.762 ^{b}	1.778 ^{ef}	0.782 ^{f}	0.221 ^{ef}	0.527 ^{de}	1.623 ^{d}	1.160 ^{c}
	P. eryngii	1.921 ^{cd}	1.167 ^{g}	0 ^{i}	0.140 ^{jk}	0.176 ^{ij}	0.402 ^{ij}	0.919 ^{e}
42	*L. edodes*	1.873 ^{de}	2.770 ^{a}	2.330 ^{a}	0.151 ^{ij}	0.326 ^{gh}	2.963 ^{a}	1.379 ^{a}
	P. citrinopileatus	1.962 ^{cd}	1.693 ^{f}	0.711 ^{f}	0.156 ^{ij}	0.445 ^{f}	2.395 ^{b}	1.138 ^{c}
	P. eryngii	1.831 ^{de}	2.248 ^{d}	0 ^{i}	0.121 ^{k}	0.146 ^{j}	0.054 ^{k}	1.003 ^{d}
	SEM	0.0196	0.0215	0.0164	0.0026	0.0090	0.0184	0.0047
Effects								
	IP	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Fungi	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	IP × Fungi	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Values with different letters within a column are significantly ($p < 0.05$) different. IP, incubation period (day); Lacc, laccase; MnP, manganese peroxidase; LnP, lignin peroxidase; CMCase, carboxymethyl cellulase; FAEs, feruloyl esterases.

The cellulolytic enzymes avicelase, and CMCase presented the same kinetics for all fungi strains, showing a peak at 14 days of IP for *L. edodes* and *P. eryngii* and 28 days for *P. citrinopileatus*. The highest activities were once again detected for *L. edodes* (0.58 and 1.51 U g^{-1} DM, for avicelase and CMCase, respectively). For the enzymes involved in hemicellulolysis, xylanase and FAEs, production peaks were attained at 42 days of IP for both *L. edodes* and *P. citrinopileatus* while for *P. eryngii* the same trend was observed for FAEs but not for xylanase, with a peak at 14 days. Again, *L. edodes* showed a higher production of these enzymes (2.96 and 1.38 U g^{-1} DM for xylanase and FAEs, respectively).

These results are consistent with the data presented in Table 1. In fact, the treatment with *L. edodes* had the higher degradability of lignin (at 42 days of IP), consistent with the higher activity values for all enzymes, although there were differences within the period in which the activity peak was achieved. As it has been previously reported [33], lignin degradation is the result of a synergistic engagement of several enzymes that promote ligninolysis efficiency, depending on the fungal strain. In order to better explain this variability, the experimental data were submitted to a PCA analysis (Figure 3).

Eigenvalues from the PCA indicated that the first two principal components (PC) accounted for 83.6% of the total data variance (PC1: 62.7% and PC2: 20.9%). Axe PC1 was positively correlated to NDF, ADFom, and lignin content, and negatively with the IVOMD, C/L ratio, enzymes involved in ligninolysis (Lacc, MnP, and LnP), and hemicellulolysis (Xylanase and FAEs). Axe PC2 was positively correlated with cellulose, and negatively with hemicellulose, CP content and enzymes involved in cellulolysis (avicelase and CMCase). PCA showed that treatment with *P. eryngii* is correlated with a high content of cellulose, NDF, ADFom, and lignin, *P. citrinopileatus* is correlated with a higher production of cellulolytic enzymes but inversely correlated with the HC content (longer IP, lower HC content), and *L. edodes* is correlated with a high ratio of C/L, a higher IVOMD, and a higher

production of enzymes involved in ligninolysis and hemicellulolysis, that increased the delignification process.

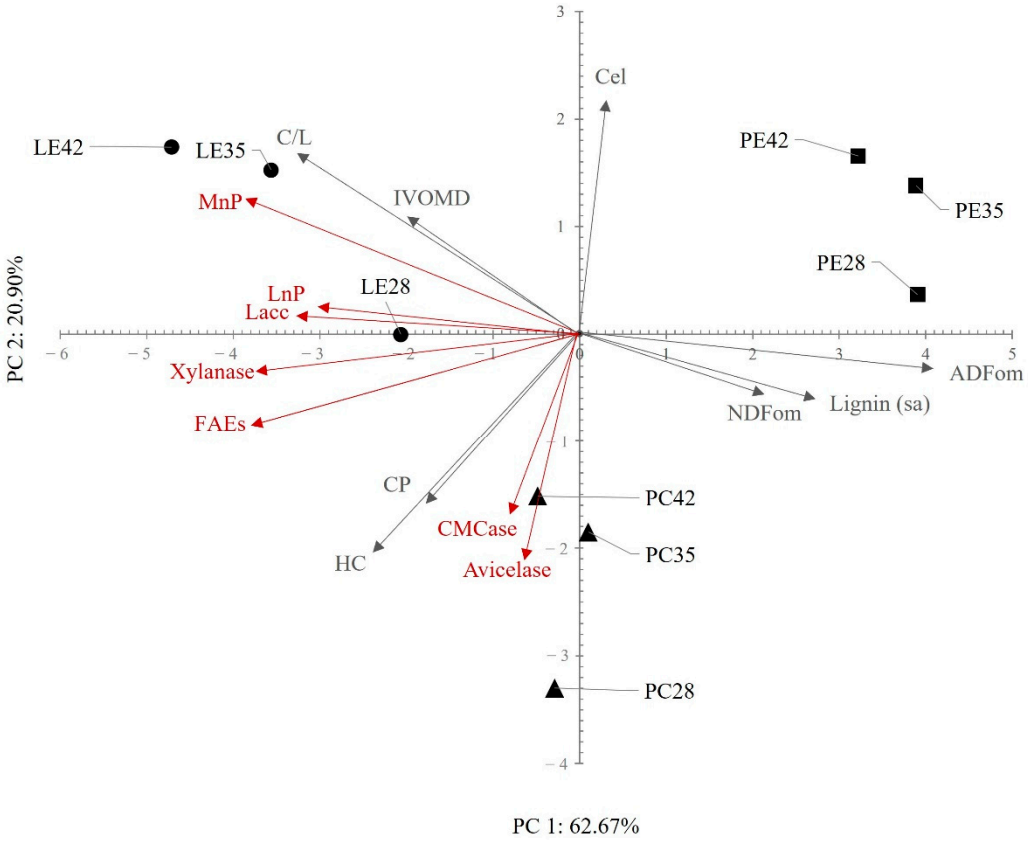

Figure 3. Principal component analysis (PCA) of fungal and time incubation influence on the analyzed parameters in treated GS (Lacc, laccase; MnP, manganese peroxidase; LnP, lignin peroxidase; CMCase, carboxymethyl cellulase; FAEs, feruloyl esterases; CP, crude protein; NDFom, neutral detergent fiber; ADFom, acid detergent fiber; HC, hemicellulose; Cel, cellulose; IVOMD, *in vitro* organic matter digestibility; C/L, cellulose/lignin (sa) ratio; LE, *L. edodes*; PC, *P. citrinopileatus*; PE, *P. eryngii*; 28, 35 and 42 days).

Changes within the structure of the cell wall of GS were also observed microscopically, by analyzing the surface morphology and micro-morphological structures of GS by scanning electron microscopy (SEM) (Figure 4).

The autoclaved GS (Figure 4(a1)) showed a tight vascular structure, and the surface was relatively smooth and compact without any dumps, while the surface of the treated GS (Figure 4(b1)) was partially damaged by fungi, showing that the dense structure was loosened and became more permeable, and it is possible to visualize a darker structure in the center of the stalk, possibly a structure belonging to the xylem channels. Magnified images show that in the untreated GS (Figure 4(a2)), a cellular organization pattern with sets of long horizontal cells alternated by rounded and shorter cells can be observed. On the other hand, in the treated GS (Figure 4(b2)), images show that the organization is no longer visible, and it is possible to observe the degradation of the cell wall, showing that the GS no longer has a complete fiber structure. Data presented in this study indicated that during the treatment with WRF, lignin contents generally decrease with the increase of the IP, and that is corroborated in studies published by other authors [7,54,55]. One of the reasons for longer incubation periods is the potential outcome of higher delignification, as lignin is not potentially degraded during primary fungal growth due to the presence of other lignocellulosic components that limit the physical contact between enzymes and lignin [55]. However, the structural effects that WRF can enhance in the substrates depend

on the characteristics of the substrate, the fungal strain, and the incubation period [22,35]. It should also be mentioned that the availability of soluble nutrients during the initial stages of incubation might enhance the utilization of these nutrients for mycelium growth, and only after the synthesis of extracellular enzymes will they be stimulated. In this way, longer incubation periods may reflect the fact that nutritional scarcity may trigger the synthesis of ligninolytic enzymes [56]. In this way, longer incubation periods are necessary to better understand the correlation between delignification and digestive utilization and to observe a potentially more pronounced beneficial association between them. In addition, future animal performance studies should be performed to evaluate the potential of including treated GS in animal feeding.

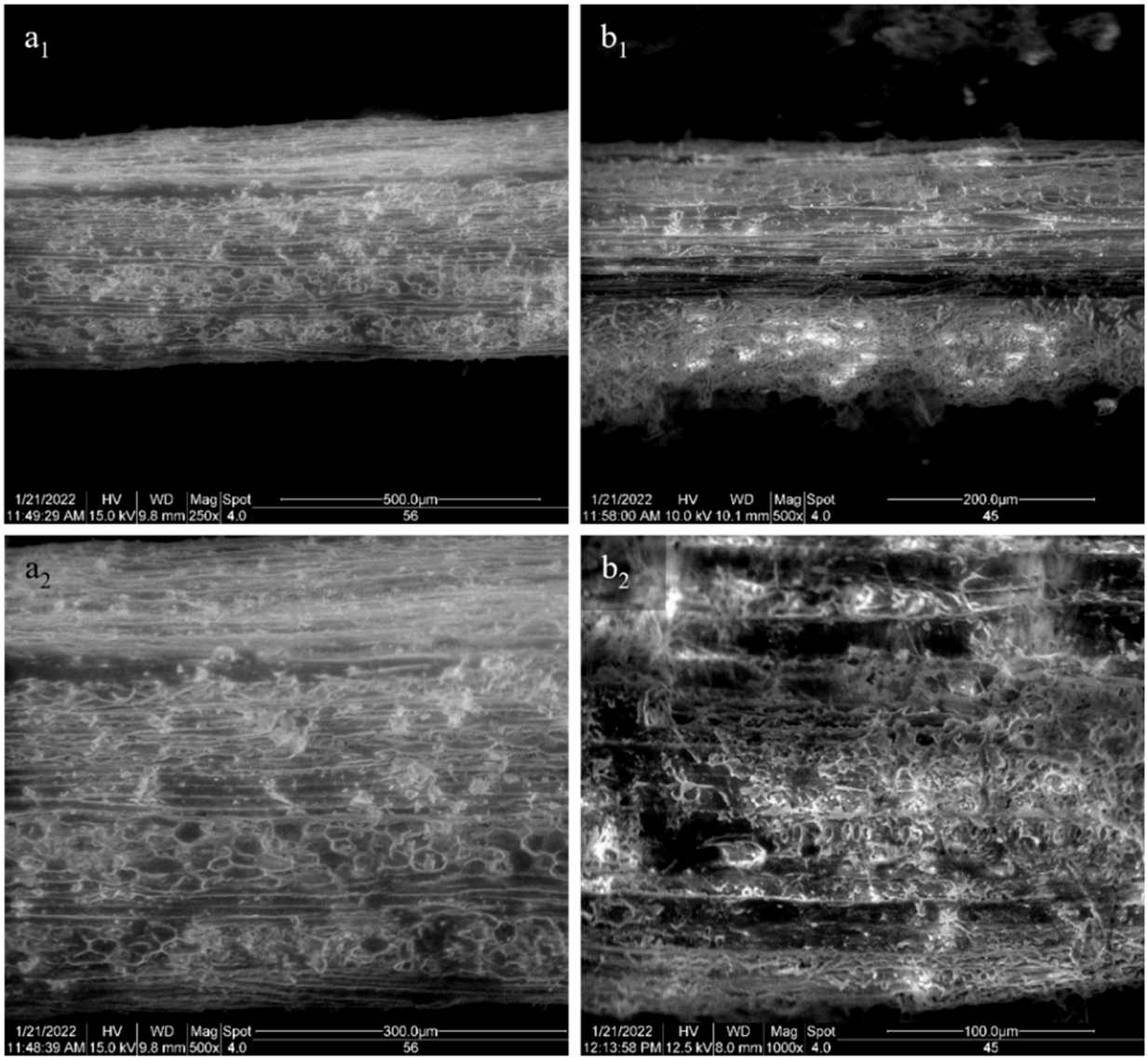

Figure 4. Scanning electron microscopy (SEM) photomicrographs of GS samples: untreated (**a1**,**a2**) and treated (**b1**,**b2**) after 42 days *L. edodes* treatment.

4. Conclusions

The present study showed that the incubation of grape stalks in solid state fermentation with white-rot fungi, in general, resulted in a decrease in the cell wall fractions, an increase in the crude protein content and *in vitro* organic matter digestibility. The white-rot fungus *L. edodes* was the most effective treatment due to their selectivity for degrading lignin and improving the *in vitro* organic matter digestibility. Based on the experimental data, 42 days

was the best incubation period for the treatment of grape stalks, but longer incubation periods should be tested to understand its correlation with the delignification process and loss of nutrients.

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