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Research article

Biochemical characteristics and *in vitro* degradability of sunflower seed hull and wheat straw treated with *Pleurotus ostreatus* and *Ganoderma sessile*

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Abstract

Importance of the work: Exploring sustainable uses for regional agricultural residues can address the need for eco-friendly solutions in livestock feeding.

Objectives: To evaluate the potential of *Pleurotus ostreatus* and *Ganoderma sessile* to transform sunflower seed hulls (SSH) and wheat straw and to enhance their *in vitro* digestibility.

Materials and Methods: SSH and wheat straw were treated with *Pleurotus ostreatus* (strains A01, PPWarm, P04, P0761, P07) and *Ganoderma sessile* strain E47. Colonization time, enzyme activity, phenolic compounds content, reducing sugar content, fiber composition and *in vitro* digestibility (IVD) were assessed, along with the impact of the inoculum formulation on these variables.

Results: Fungal strains colonized substrates within 10–25 d. Laccase activity was in the ranges 2,266–4,621 U/kg in SSH and 1,135–7,279 U/kg in wheat straw. Peroxidase activity was in the range 0–490 U/kg. In SSH, reducing sugars increased, with *G. sessile* E47 producing a 164% increase ($p < 0.001$). In contrast, in wheat straw, reducing sugars decreased, with *P. ostreatus* P04 reducing the content by 64% ($p < 0.001$). The phenolic and triterpenoid contents decreased across all treatments. Fiber content changes were substrate-dependent—in the SSH, neutral detergent fiber and acid detergent fiber fractions, increased by 14.1–21.6%, while in the wheat straw, changes were minimal. IVD remained unchanged for most treatments, except for *P. ostreatus* PPWarm (-20.7%, $p = 0.013$) and *G. sessile* E47 (-60.9%, $p < 0.001$) on SSH. Inoculum formulation without grains altered mycelial effects on substrate degradation, increasing SSH digestibility by 70.5% ($p = 0.003$) after treatment with *P. ostreatus* A01.

Main finding: The fungal treatments modified the SSH and wheat straw composition, with digestibility improvement critically dependent on inoculum type and substrate.

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Introduction

Securing sufficient green fodder for animals is a worldwide challenge in modern agriculture, with forage scarcity being exacerbated by resource competition among food, fodder and fuels, increasing pressure on food prices and availability (Sufyan et al., 2022). Addressing this issue requires innovative solutions to optimize agricultural biomass use for livestock. For example, lignocellulosic residues, such as sunflower seed hulls (SSH) and wheat straw, are underutilized resources with potential as sustainable animal feed (Sharma and Arora, 2013; van Kuijk et al., 2015a).

SSH and wheat straw are promising residues in the Southwest Buenos Aires region of Argentina. In 2023, global trade of sunflower seeds reached 20.36 million metric tons, mainly for sunflower oil (Nurromat et al., 2024). Sunflower seeds consist of 20–30% hulls, which typically are removed before oil extraction due to their negative impact on processing and product quality (Isobe et al., 1992). Consequently, most SSH is stockpiled outdoors, landfilled or burned, with wind-driven dispersal of fine particles causing visibility hazards and traffic disruptions (Casoni et al., 2019). Wheat straw, produced at an estimated 1.3 kg/kg of wheat grain, yields about 920 million t annually worldwide (Pereira et al., 2017). As much as 85 % of this straw is burned *in situ* to clear fields, contributing markedly to air pollution, greenhouse-gas emissions and soil degradation (Montero et al., 2018).

Despite their abundance, SSH and wheat straw each have a high lignin content that limits ruminal digestibility by impeding microbial access to cellulose and hemicellulose (Agosin et al., 1986; Arora and Sharma, 2009; van Kuijk et al., 2015b). Lignin acts as a physical and chemical barrier to rumen microbes, severely constraining dry matter digestibility (van Kuijk et al., 2015b). White-rot fungi offer a sustainable treatment by selectively degrading lignin through extracellular enzymes, with laccases generating free radicals that destabilize lignin bonds, while peroxidases (LiP, MnP) oxidize non-phenolic lignin units via hydrogen peroxide (Wang et al., 2018). This bioprocess increases accessibility to cellulose and hemicellulose, improving overall digestibility. However, treatment outcomes vary widely with strain and substrate, underscoring the need to tailor protocols to local residues (Wang et al., 2021; Sufyan et al., 2022). No published studies have investigated SSH biological enhancement as forage, highlighting a critical research gap.

Selective degradation of lignin depends on the interaction between time, strain and substrate. Generally, selective lignin removal occurs during early colonization—before fruiting body formation and cellulose consumption (Kirk and Moore, 1972; van Kuijk et al., 2015a)—although significant degradation has been observed after up to 20 wk of incubation (Okano et al., 2005). Incubation duration also influences nutritional parameters, including crude protein and fiber fractions—neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), hemicellulose and cellulose (Khan et al., 2015). Furthermore, degradation selectivity is affected markedly by species and strain differences, lignin structure, substrate composition, moisture and particle size (Akin et al., 1993; Zadrazil and Puniya, 1995; Tripathi et al., 2008; Khan et al., 2015). For example, while *Pleurotus ostreatus* reduces ADL, ADF and NDF across various substrates, the extent of degradation depends on substrate type and conditions (Khan et al., 2015).

Pleurotus ostreatus and *Ganoderma sessile* combine robust growth under solid-state fermentation (SSF) with high extracellular enzyme production and proven capacity to bioconvert complex lignocellulosic substrates (Curvetto et al., 2002; Bidegain et al., 2015). Their widespread cultivation, commercial availability and regulatory acceptance as safe for animal feed make them practical candidates for large-scale feed enhancement.

The current study hypothesized that selected strains of *Pleurotus ostreatus* and *Ganoderma sessile* could selectively degrade lignin in SSH and wheat straw, thereby enhancing their *in vitro* digestibility. Based on this hypothesis, the aim of this study was to evaluate five strains of *P. ostreatus* and one strain of *G. sessile* to transform SSH and wheat straw and improve digestibility. Specifically, the objectives were: 1) to identify which strain-substrate combination maximized lignin degradation and *in vitro* digestibility; and 2) to assess how using wheat grain as a carrier affected the performance of *P. ostreatus* A01 on both substrates.

Materials and Methods

Mushroom strains

Commercial strains of edible fungi were evaluated, consisting of five strains of *Pleurutus ostreatus,* A01 (INTECH, Argentina), PPWarm (Gertisem, Argentina), P04, P0761 and P07 (Autonomous University of Tamaulipas, Mexico) and one strain of *Ganoderma sessile* E47 (University of Guelph, Canada), classified as Generally Recognized as Safe (GRAS) and maintained in the culture collection of the Laboratory of Edible and Medicinal Mushroom Biotechnology at the Center for Renewable Natural Resources of the Semi-arid Zone (CERZOS), Argentina (Curvetto et al., 2002; Bidegain et al., 2015). These strains are commercially distributed for cultivation intended for human consumption.

Substrate fungal treatment

Wheat straw and SSH were autoclaved separately at 121°C for 150 min after soaking to 60% moisture content. Bags with 500 g of sterilized substrate and 50 g of spawn on wheat grains were incubated at 28°C in darkness until fully colonized. Each strain-substrate combination had eight replicates. Controls contained uncolonized wheat grains and were handled identically. A small-scale treatment with *P. ostreatus* A01 and SSH as the spawn carrier was conducted with six replicates. After full colonization, the incubation time was recorded, and the substrate was removed, homogenized and fractionated for analysis.

Enzyme activity determination

Enzyme activity was determined from homogenized treated substrate extracts using citrate buffer (100 mM, pH 4.5). Laccase and peroxidase activities were measured colorimetrically according to Postemsky et al. (2019). To quantify laccase activity, the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 25°C and 420 nm (Molar extinction coefficient at 420 nm = 3.6×10^4 1/M cm) was monitored for at least 2 min. The activity of peroxidases was measured similarly; however, a final concentration of 0.1 mM H_2O_2 was added to the reaction mixture. Peroxidase activity was adjusted for background laccase activity. The results were expressed in units of enzymatic activity per kilogram of dry substrate (U/kg), where 1 unit of enzymatic activity represents the oxidation of 1 mmol of ABTS per minute.

Chemical analysis

Reducing sugars were quantified using the 3,5-dinitrosalicylic acid (DNS) method. Extracts from homogenized substrate were mixed with DNS reagent, boiled and then their absorbance was measured at 550 nm using a spectrophotometer (Genesys 20; Thermo Scientific; USA).

NDF and ADF were analyzed using an Ankom 200 Fiber Analyzer (ANKOM Technology; USA). The ADL content was determined by extracting the residues remaining after acid-detergent extraction with 72% sulfuric acid and adjusting for the ash content. Hemicellulose (HC) was calculated as the difference between NDF and ADF, while the cellulose content was calculated as the difference between ADF and lignin.

The ash content was determined by incinerating the sample in a muffle furnace (Thermolyne F6020C-33-80; Thermo Scientific; USA) at 500°C for 24 hr.

The phenolic contents in both the aqueous and ethanolic extracts obtained from the substrates were determined using the Folin-Ciocalteu technique adapted for 96-well microplates, with gallic acid serving as the standard (Magalhães et al., 2010). The triterpenoid content was assessed in ethanolic extracts using the vanillin-perchloric acid method adapted to 96-well microplates and compared with an ursolic acid (U6753; Sigma; USA) standard (Bidegain et al., 2019) using a spectrophotometer (Synergy-HT; BioTek; USA).

In vitro digestibility assay

The biotransformed substrate was dried in an oven at a temperature below 60°C for at least 48 hr and subsequently ground (2 mm grid size). The fresh and dry weights of the samples were measured.

The analysis was carried out by the Animal Nutrition Department at the National University of La Pampa in Santa Rosa, Argentina, utilizing the Tilley and Terry two-stage *in vitro* digestibility method (Tilley and Terry, 1963).

Statistical analysis

Statistical analysis was carried out using the Python software (version 3.12.1; Python Software Foundation, Wilmington, DE, USA) along with the library SciPy 1.11.1 (Virtanen et al., 2020). Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test for comparisons among means.

Results and Discussion

Mycelium growth and substrate colonization of mushroom strains

Complete colonization of the substrate by fungal mycelia (defined as when the mycelia fully covered the substrate) was used as the endpoint to ensure that strains were evaluated at equivalent growth stages. All the strains evaluated achieved complete colonization of SSH within 10–14 d, although greater variability was observed in wheat straw, with complete colonization occurring within 12–25 d (Table 1). The *G. sessile* E47 strain and the *P. ostreatus* PPWarm strain had the fastest colonization rate in SSH (10 d), while in wheat straw, the *G. sessile* E47 strain and the *P. ostreatus* P07 strains achieved complete colonization in 12 d and 17 d, respectively. These colonization times are similar to those reported in other studies using SSH (Curvetto et al., 2002; Bidegain et al., 2015) or wheat straw (Labuschagne et al., 2000; Ćilerdžić et al., 2018). Notably, shorter colonization times have practical implications, as they reduce the overall production time and thus offer a commercial advantage, in addition to lowering the risk of contamination by competing organisms.

Table 1 Time required for complete colonization (days) of sunflower seed hulls (SSH) and wheat straw by different strains of *Pleurotus ostreatus* and *Ganoderma sessile.*

Species/Strain	Time to complete colonization (d)	
	Sunflower seed hulls	Wheat straw
Pleurotus ostreatus		
P04	14	21
PPWarm	10	16
P0761	12	25
P07	12	17
A01	11	19
Ganoderma sessile		
E47	10	12

Laccase and peroxidase enzyme activity

Ligninolytic enzyme activity (Fig. 1) was variable among strains and influenced by the substrate source. Laccase activity was in the ranges 2,266–4,621 U/kg in SSH and 1,135–7,279 U/kg in wheat straw. The strains with the highest activity were A01 in SSH and E47 in wheat straw, although in the latter case, the laccase activity observed in A01 was very similar. The variation between samples of a treatment was lower in SSH. In the case of wheat straw, much larger ranges were observed. For example, for P04, laccase activity was in the range 2,100–15,900 U/kg. The lower variability in laccase activity in SSH may have been due to its homogeneous composition, ensuring consistent fungal growth and enzyme production. In contrast, wheat straw's structural heterogeneity may have increased the variability among strains and replicates. Sampling during enzyme extraction could further amplify these differences by causing uneven biomass or enzyme distribution.

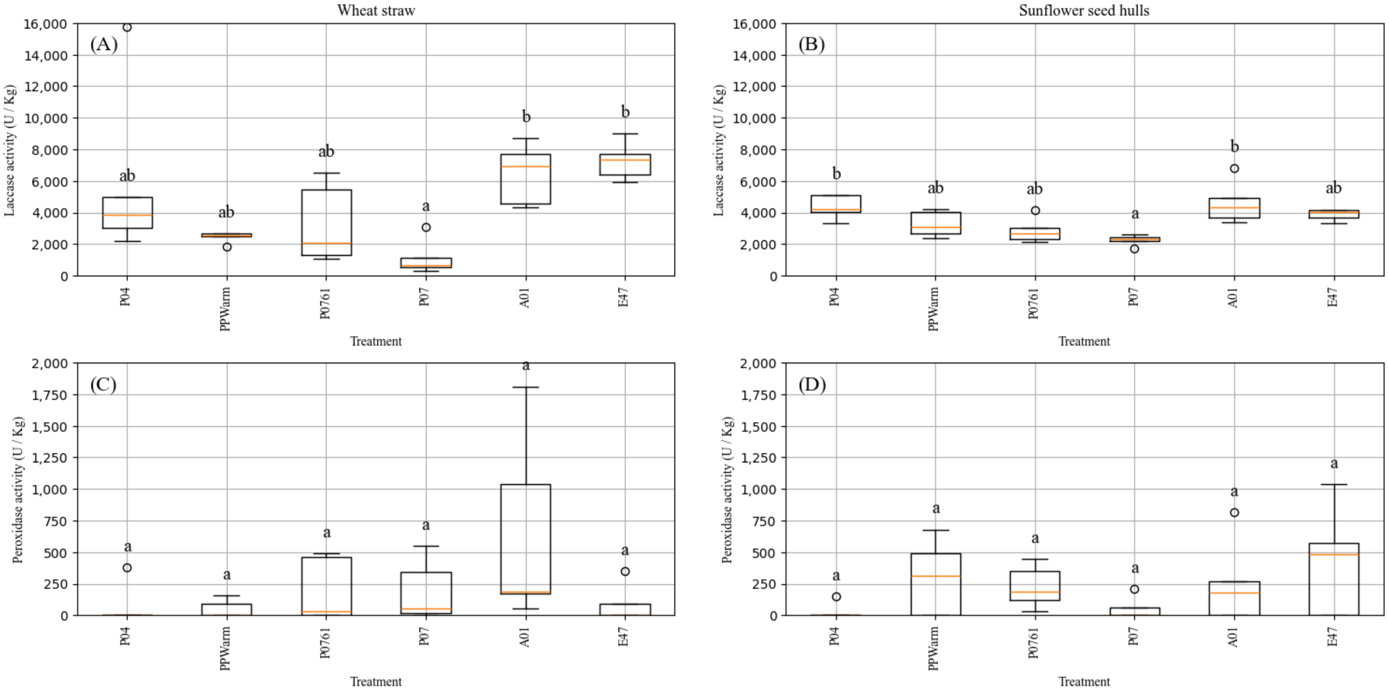

Fig. 1 Effects of different fungal strains of *Pleurotus ostreatus* (P04, PPWarm, P0761, P07, A01) and *Ganoderma sessile* (E47) on laccase and peroxidase activities in wheat straw (A and C, respectively) and sunflower seed hulls (B and D, respectively). Boxplots show the median (orange line), interquartile range (box) and extreme values within 1.5 times interquartile range (error bars). Outliers are represented by circles. Treatments with different lowercase letter within group are significantly different ($p < 0.05$, Dunn's test). One unit of enzymatic activity represents the oxidation of 1 mmol 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) per minute.

For the peroxidases, a large number of samples showed no activity, although in general, there was higher activity in treatments performed on SSH. Peroxidase activity was in the ranges 0–287 U/kg in wheat straw and 0–490 U/kg in SSH, with the strains A01 and E47 showing the highest activity in each substrate, respectively.

The observed variability in ligninolytic enzyme activity levels across samples, treatments and substrates underscored the complex interplay between fungal strains and their cultivation conditions. Notably, the current findings revealed a considerable range in laccase activity, indicative of the diverse enzymatic potential exhibited by different strains and their adaptability to distinct substrates. The pronounced variability in peroxidase activity could be attributed to several factors, as described by Giardina et al. (2000). Peroxidase activity is known to be highly sensitive to external conditions, such as substrate type, ion concentration, and pH, making it inherently variable across different samples. Additionally, peroxidase activity often occurs within narrow time windows during fungal development. In the current study, beyond the variability between strains and substrates, the observed differences within the same treatment could have been due to a lack of synchronization in the production phase among replicates. Even slight delays or advances in enzyme production could result in a failure to detect activity at the time of sampling, leading to the observed negligible activity in some samples. This hypothesis aligned with other research suggesting that enzyme expression profiles can be influenced by the stage of fungal growth and substrate availability (van Kuijk et al., 2015b).

The current results also underscored the importance of substrate specificity in influencing enzyme activity. For example, treatments involving SSH generally exhibited higher peroxidase activity than those with wheat straw substrates, suggesting that the composition and structure of the substrate could substantially impact enzyme expression and activity, reflecting the complex interactions between fungal physiology and substrate biochemistry (Giardina et al., 2000; Postemsky et al., 2019; Vela Gurovic et al., 2023).

Effect on nutritional content and in vitro digestibility

The degradation of organic matter during SSF poses a major challenge in these kinds of biological treatments (Akin et al., 1993). Fungi breakdown organic matter, producing CO_2 and water through respiration, thereby resulting in a substantial weight loss. The extent of this issue varies depending on the biomass source; while some abundant residues may experience a compensatory effect due to their low economic value (Khan et al., 2015), others may face more pronounced challenges.

Tables 2 and 3 present the effects of mushroom growth on organic matter and fiber content and IVD. The strains P0761 and P07 significantly decreased organic matter content in wheat straw by 9.9% ($p < 0.001$) and 6.9% ($p = 0.024$), respectively. On the other hand, fungal growth in SSH did not reduce its organic matter content, implying a lesser degree of substrate degradation.

Table 2 Chemical composition (g/kg DM) and *in vitro* digestibility of sunflower seed hulls after treatment with different fungal strains of *Pleurotus ostreatus* (P04, PPWarm, P0761, P07, A01) and *Ganoderma sessile* (E47), compared with untreated control.

	Treatment							
	Control	P04	PPwarm	P0761	P07	A01	E47	p-values
105°C dry matter (g/kg)								
Organic matter	839 ± 40^{a}	831 ± 30^{a}	840 ± 31^{a}	848 ± 37^{a}	826 ± 45^{a}	823 ± 31^{a}	880 ± 44^{a}	0.274
Neutral detergent fiber	691 ± 16^{b}	744 ± 28^{a}	744 ± 14^{a}	742 ± 9^{a}	737 ± 15^{a}	730 ± 11^{a}	741 ± 22^{a}	0.004
Acid detergent fiber	487 ± 18^{b}	558 ± 28^{a}	556 ± 15^{a}	559 ± 9^{a}	566 ± 26^{a}	572 ± 33^{a}	592 ± 22^{a}	< 0.001
Acid detergent lignin	202 ± 5^{a}	228 ± 34^{a}	228 ± 9^{a}	239 ± 27^{a}	231 ± 18^{a}	229 ± 20^{a}	239 ± 22^{a}	0.195
Hemicellulose	204 ± 3^{a}	186 ± 5^{ab}	188 ± 9^{a}	182 ± 2^{ab}	171 ± 27^{abc}	158 ± 33^{bc}	149 ± 13^{c}	0.002
Cellulose	285 ± 18^{b}	329 ± 16^{a}	328 ± 14^{a}	320 ± 7^{a}	335 ± 22^{a}	343 ± 32^{a}	353 ± 16^{a}	0.002
In vitro digestibility	371 ± 43^{c}	329 ± 43^{bc}	294 ± 35^{b}	332 ± 26^{bc}	349 ± 22^{bc}	378 ± 18^{c}	145 ± 35^{a}	< 0.001

Values (mean ± SD) within a row with different lowercase superscripts are significantly different ($p < 0.05$).

Table 3 Chemical composition (g/kg DM) and *in vitro* digestibility of wheat straw after treatment with different fungal strains of *Pleurotus ostreatus* (P04, PPWarm, P0761, P07, A01) and *Ganoderma sessile* (E47), compared with untreated control.

	Treatment							
	Control	P04	PPWarm	P0761	P07	A01	E47	*p* values
105°C dry matter (g/kg)								
Organic Matter	941 ± 9^{a}	915 ± 23^{ab}	899 ± 46^{abc}	848 ± 38^{c}	876 ± 34^{bc}	907 ± 38^{ab}	927 ± 11^{ab}	< 0.001
Neutral detergent fiber	630 ± 15^{a}	628 ± 43^{a}	608 ± 60^{a}	618 ± 52^{a}	586 ± 79^{a}	610 ± 22^{a}	585 ± 24^{a}	0.500
Acid detergent fiber	345 ± 22^{a}	366 ± 34^{a}	351 ± 37^{a}	351 ± 34^{a}	327 ± 55^{a}	348 ± 14^{a}	334 ± 18^{a}	0.574
Acid detergent lignin	60 ± 3^{a}	57 ± 7^{a}	52 ± 12^{a}	58 ± 6^{a}	57 ± 11^{a}	59 ± 8^{a}	65 ± 10^{a}	0.275
Hemicellulose	286 ± 10^{a}	264 ± 15^{ab}	256 ± 23^{ab}	267 ± 19^{ab}	259 ± 30^{ab}	262 ± 11^{ab}	251 ± 8^{b}	0.041
Cellulose	284 ± 22^{a}	308 ± 27^{a}	300 ± 26^{a}	293 ± 30^{a}	270 ± 48^{a}	289 ± 8^{a}	269 ± 14^{a}	0.181
In vitro digestibility	642 ± 17^{ab}	602 ± 38^{ab}	671 ± 75^{b}	627 ± 57^{ab}	632 ± 62^{ab}	604 ± 45^{ab}	516 ± 68^{b}	0.038

Values (mean ± SD) within a row with different lowercase superscripts are significantly different ($p < 0.05$).

Notably, the strains P0761 and P07 had lower laccase activity at the time of sampling, suggesting that the peak may have occurred earlier. These results highlighted the importance of considering the timing of enzyme activity during incubation, as peak activity may not coincide with the sampling point, potentially obscuring the link between enzymatic profiles and lignocellulose degradation. A similar situation was observed with the strains A01 and E47, which exhibited high laccase and peroxidase activity, yet this was not associated consistently with greater reductions in fiber fractions, reinforcing that the enzymatic profiles at sampling may not have fully reflected prior degradation events.

Based on the fiber content analysis, it was apparent that the fungal behavior varied depending on the substrate. For example, with the wheat straw, characterized by a lower lignin and higher reducing sugar content than the SSH, there was a well-distributed consumption of fibers and soluble compounds. This led to no significant variation in the relative composition of the biotransformed substrate compared to the control, except for the *G. sessile* E47 strain, which displayed a preference for hemicellulose consumption. The preferential degradation of HC by fungi has been reported in several studies on wheat straw (Agosin et al., 1986), rice straw (Karunanandaa and Varga, 1996) and Japanese red cedar (Okano et al., 2005), among others. Furthermore, there was no discernible selective degradation of lignin, as evidenced by the absence of a relative increase in the cellulose content and a decrease in the lignin content.

On the other hand, in SSH there was a significant increase observed in both the NDF and ADF fractions, indicating a fungal preference for consuming the more readily available nutrients in the soluble phase. The increase was in the range 14.1–17.4% for the SSH treated with *P. ostreatus* strains, and reached 21.6% ($p < 0.001$) for the *G. sessile* strain. Additionally, there was a relative increase in the cellulose content, up to 20.3% for *P. ostreatus* A01 ($p = 0.001$) and 23.9% for *G. sessile* E47 ($p < 0.001$)*,* suggesting greater degradation of the remaining components. Furthermore, significant decreases of 22.5% ($p = 0.004$) and 27.0% ($p = 0.001$) in the HC content were noted with the *P. ostreatus* A01 and *G. sessile* E47 strains, respectively, demonstrating a preferential consumption of this fraction.

Notably, IVD remained unchanged for most treatments, except for PPWarm (-20.7%, $p = 0.013$) and *G. sessile* E47 (-60.9%, $p < 0.001$) on SSH. Furthermore, the E47 strain had a marked negative impact on IVD, likely because this strain produces antimicrobial compounds that affect the rumen microbiota, as it has been reported that *Ganoderma* sp. produced antimicrobial compounds (Gao et al., 2005). This information is interesting and deserves further investigation; however, it also suggests that care should be used when feeding *Ganoderma* to ruminants, as it could negatively impact digestibility. SSF with *Ganoderma* has been reported to improve the digestibility of a range of substrates (Misra et al., 2007; Shrivastava et al., 2012), so this could be a strain-specific occurrence.

Reducing sugar content

Differential behavior was observed in the contents of the reducing sugars, depending on the substrate used (Table 4). In SSH, all strains increased the contents of reducing sugars compared to the control, with strain *G. sessile* E47 having the greatest increase (+164%, $p < 0.001$). In wheat straw, all strains decreased the contents of reducing sugars, with *G. sessile* E47 having the smallest reduction (-18.6%, $p \geq 0.05$). On the other hand, the strain *P. ostreatus* P04 reduced considerably the content of reducing sugars in wheat straw by 64% ($p < 0.001$).

Table 4 Concentrations of soluble reducing sugars, water soluble phenolic compounds and triterpenoids (mg/g) in sunflower seed hulls and wheat straw after treatment with different fungal strains of *Pleurotus ostreatus* (P04, PPWarm, P07, P0761, A01) and *Ganoderma sessile* (E47), compared with untreated control.

Treatment	Sunflower seed hulls			Wheat straw		
	Soluble reducing sugars (mg/g)	Water soluble phenolic compounds (mg/g)	Triterpenoids (mg/g)	Soluble reducing sugars (mg/g)	Water soluble phenolic compounds (mg/g)	Triterpenoids (mg/g)
Control	14.4 ± 5.0^{d}	1.38 ± 0.26^{b}	19.25 ± 2.29^{d}	97.1 ± 12.3^{a}	4.22 ± 1.11^{b}	12.02 ± 2.30^{d}
P04	24.0 ± 2.3^{bcd}	0.73 ± 0.21^{a}	5.29 ± 0.59^{c}	35.0 ± 14.0^{c}	0.98 ± 0.36^{a}	4.82 ± 0.31^{ab}
PPWarm	30.7 ± 6.5^{ab}	0.60 ± 0.15^{a}	4.48 ± 0.42^{abc}	54.7 ± 3.0^{bc}	0.83 ± 0.55^{a}	4.68 ± 0.55^{ab}
P07	34.8 ± 4.9^{ab}	0.69 ± 0.17^{a}	3.58 ± 0.39^{ab}	45.5 ± 9.7^{bc}	0.54 ± 0.21^{a}	4.53 ± 0.41^{ab}
P0761	24.1 ± 6.0^{bcd}	0.61 ± 0.11^{a}	3.60 ± 0.57^{ab}	41.2 ± 8.9^{bc}	0.89 ± 0.36^{a}	3.55 ± 0.28^{a}
A01	31.1 ± 2.0^{abc}	0.61 ± 0.18^{a}	3.48 ± 0.38^{b}	57.8 ± 9.1^{b}	0.90 ± 0.25^{a}	5.16 ± 0.69^{b}
E47	37.3 ± 6.1^{a}	0.61 ± 0.22^{a}	5.81 ± 1.34^{c}	79.3 ± 13.1^{a}	1.16 ± 0.24^{a}	8.26 ± 0.79^{c}
p values	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Values (mean ± SD) within a column with different lowercase superscripts are significantly different ($p < 0.05$).

During the SSF process, fungi carry out two opposing processes: substrate degradation to produce reducing sugars and the consumption of these sugars for energy. The relationship between these processes determines the final content of reducing sugars in the substrate after fermentation. It has been observed that when the substrate has a high initial concentration of reducing sugars, the fungus tends to preferentially consume these sugars for energy, leading to a reduction in their content. On the contrary, when the substrate has a low concentration of reducing sugars, substrate degradation predominates, resulting in an increase in the concentration of reducing sugars during SSF. For example, substrates initially containing low levels of reducing sugars (such as an olive pomace-to-pistachio ratio of 90:10 treated with *P. ostreatus*) had an increase in reducing sugar content from 0.66% to 2.04% (Eliopoulos et al., 2022c), while mixtures of pomace with *Lathyrus* pericarp had an increase from 0.36% to 0.72% when also fermented with *P. ostreatus* (Eliopoulos et al., 2022b). However, substrates with initially high sugar contents experience a reduction. For example, in industrial hemp, the content was reduced from 3.54% to 1.36% (Eliopoulos et al., 2022a) and in fruit and vegetable residues from 26.5% to 4.3% (Ibarruri et al., 2021), when treated with *P. ostreatus* and *Rhizopus* sp., respectively. These changes in the contents of reducing sugars are relevant not only due to their implications for the nutritional content of the substrate but also because different sugars can influence cellulase expression, affecting cellulose degradation (Vela Gurovic et al., 2023). Likewise, it has been reported that the concentration of reducing sugars during SSF varies over time, which probably explains the differences in the final content of reducing sugars in the analyzed substrates (Eliopoulos et al., 2022b).

Triterpenoid and phenolic compound contents

Across all fungal treatments, decreases were observed in the phenolic content, as well as in the levels of triterpenoids (Table 4), compared to the original substrates. Phenolic compounds are known to inhibit bacteria in the rumen (Akin and Rigsby, 1985). Lignin degradation produces phenolic compounds, with fungi possessing phenolic acid esterases that degrade these compounds (Akin et al., 1993). Based on the current results, although phenolic compounds could have been produced from lignin degradation, the effect of their subsequent degradation was much greater, leading to an overall reduction in all treatments. This reduction in the phenolic content could have the potential to improve the nutritional value of the forage.

Particularly noteworthy was the substantial reduction in the phenolic and triterpenoid contents observed with the *P. ostreatus* P0761 strain, evident in both substrates (-55.8%, $p < 0.001$ and -81.3%, $p < 0.001$ in SSH and -78.9%, $p < 0.001$ and -70.9%, $p < 0.001$ in wheat straw, for the phenolic and triterpenoid contents, respectively). Intriguingly, despite these significant reductions, the strain also displayed relatively lower laccase and peroxidase activities by the conclusion of the process. This further underscored the complex relationship between substrate modification, enzyme activity and time. Additionally, *G. sessile* E47 reduced both the phenolic content (-55.8% in SSH $p < 0.001$, and -72.5% in wheat straw $p < 0.001$) and the triterpenoid content (-69.8% in SSH $p < 0.001$, and -31.3% in wheat straw $p < 0.001$).

Effect of nature of spawn carrier

Given the absence of observed selective lignin degradation, it was possible that the presence of wheat grains (acting as carriers of mycelia in the inoculum) might have inhibited such specificity in fungal activity. This supposition stems from the potential impact of a high inoculation rate, wherein fungal mycelia prioritize the utilization of the nutrients readily available in the grains. This hypothesis was tested by selecting *P. ostreatus* A01 based on substrate degradation, enzyme activity, and colonization dynamics. SSH treated with A01 had the highest IVD, while *P. ostreatus* PPWarm achieved the highest IVD for wheat straw. However, neither was statistically significant compared to the untreated substrate. The key factor favoring A01 was that PPWarm significantly reduced IVD in SSH. Subsequently, to elucidate the influence of grain presence on fungal behavior, a fungal spawn was developed using the less nutrient-rich SSH as the carrier (Table 5).

The grains in the inoculum significantly contributed to the digestible nutrients in the rumen. Based on the *in vitro* digestibility data, the digestibility of wheat straw without grains was 57.1%, while with grains, it increased to 64.2% ($p \geq 0.05$). The effect was more pronounced for SSH, where digestibility increased from 13.9% without grains to 37.1% ($p = 0.003$) with grains. When using the alternative inoculum, the effect on the nutritional content and digestibility of wheat straw and SSH varied depending on the substrate. In wheat straw, which had the lowest lignin content, there was selective degradation of HC (-33.9%, $p < 0.001$) and lignin (-39.2%, $p < 0.001$), resulting in a significant 38% increase in cellulose content. However, a 20.3% ($p = 0.001$) decrease in digestibility was also observed, likely due to the increase in the ADF fraction. On the other hand, in SSH, the A01 strain also selectively degraded lignin (-27.4%, $p < 0.001$) and HC (-14.4%, $p < 0.001$). This degradation profile was substrate-dependent, affecting not only the type of components degraded but also the resulting impact on *in vitro* digestibility, which depends on the final composition of the biotransformed substrate, as seen in the contrasting effects on wheat straw and SSH.

Based on the conclusions drawn from the comparison of wheat straw biotransformed with *P. ostreatus* A01, the absence of wheat grains significantly affected IVD. Specifically, IVD was 24.7% lower ($p < 0.001$) when wheat grains were not present, probably due to an 8.5% reduction in the organic matter content ($p < 0.003$). In contrast, this trend was not observed with SSH, where IVD increased by 59.4% ($p < 0.001$) in the treated samples without grains. This discrepancy could be attributed to the same mechanism previously explained regarding the reducing sugars content, where an increase was noted in SSH and a decrease in wheat straw. The absence of easily digestible wheat grains, combined with the more fibrous nature of SSH, which contains fewer readily available nutrients, results in enhanced degradation of the material by the mycelia. This process led to the production of easily digestible soluble compounds, which ultimately resulted in a higher level of IVD for the material biotransformed without grains compared to that biotransformed with grains, effectively compensating for the absence of wheat grain.

Conclusions

This study aimed to identify optimal strain-substrate combinations to maximize lignin degradation and improve *in vitro* digestibility of wheat straw and SSH. In addition, it provided the first biological treatment evaluation of SSH. Based on the results, none of the tested fungal strains improved

Table 5 Chemical composition (g/kg DM) and *in vitro* digestibility of sunflower seed hulls and wheat straw after treatment with *Pleurotus ostreatus* strain A01 compared with untreated control.

	Sunflower seed hulls			Wheat straw		
	Control	A01	*p* values	Control	A01	*p* values
105°C dry matter (g/kg)						
Organic Matter	834 ± 9	774 ± 13	0.056	838 ± 28	830 ± 22	0.636
Neutral detergent fiber	795 ± 25	710 ± 30	< 0.001	659 ± 21	643 ± 35	0.353
Acid detergent fiber	586 ± 20	531 ± 24	0.001	405 ± 16	475 ± 24	< 0.001
Acid detergent lignin	255 ± 6	185 ± 8	< 0.001	107 ± 12	65 ± 9	< 0.001
Hemicellulose	209 ± 6	179 ± 8	< 0.001	254 ± 10	168 ± 13	< 0.001
Cellulose	331 ± 21	346 ± 17	0.218	298 ± 24	410 ± 28	< 0.001
In vitro digestibility	139 ± 40	237 ± 36	0.003	571 ± 49	455 ± 37	0.001

Data are shown as mean ± SD values.

the *in vitro* digestibility of either SSH or wheat straw when applied with a grain-based inoculum—any apparent benefits stemmed from the grains themselves rather than the fungal activity. However, *Pleurotus ostreatus* A01 treatment of SSH with a grain-free inoculum showed an improvement in *in vitro* digestibility, partially supporting the hypothesis that selected fungal strains could enhance lignin degradation and digestibility under certain conditions. Future work should refine the inoculum formulations, extend the incubation time and validate these effects *in vivo*, particularly in terms of feed utilization, rumen fermentation and animal performance.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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