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Fungal Treatment and Wheat Straw Blend for Enhanced Animal Feed from Olive Pulp

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ABSTRACT

Amid fodder shortages and environmental concerns in Morocco, this study explores a transformative livestock feed strategy. By combining olive pulp (OP) and wheat straw (WS) treated with *Phanerochaete chrysosporium* and *Fusarium oxysporum,* we enhance digestibility and sustainability. Five mixing ratios were examined: 100% OP (OP), 75% OP and 25% WS (MOP), 50% OP and 50% WS (OPWS), 25% OP and 75% WS (MWS), and 100% WS (WS). Fungal treatment and ratios influence cellulose-lignin dynamics. MOP increased cellulose (13.1), OP showed an initial decrease (-8.51, -5.88 for *P. chrysosporium, F. oxysporum*), with cellulose rising from 4 to 8 weeks, then declining. Lignin degradation differed ($P < 0.001$), *P. chrysosporium was* efficient (24.22%±13.75 to 31.57% ± 20.65), MWS remarkable, and OPWS stable. Mixed substrates showed higher IVTD_imp (58.56% ± 16%, 54.18% ± 20%, 36.83% ± 18%), OP and WS lower (26.25% ± 11%, 14.43% ± 7.48%). Enhanced IVTD (4-12 weeks) seen, OPWS and MOP excelling, WS lower. In conclusion, this study unveils the potential of fungal-treated feed optimization through substrate composition and tailored treatment durations. By leveraging synergistic effects and optimizing treatment timelines, we enhance livestock feed sustainability while addressing waste management concerns. This comprehensive approach holds promise for achieving both nutritional and environmental goals in livestock production.

Keywords: lignin loss, cellulose improvement, digestibility, olive pulp, wheat straw, fungal treatment.

INTRODUCTION

Morocco, like many regions worldwide, grapples with a severe shortage of animal fodder due to recurring droughts and rising costs of traditional forages (Bahta and Myeki, 2021). This predicament threatens both livestock farming and food security, necessitating sustainable solutions. Simultaneously, Morocco faces environmental and economic challenges related to the management of olive oil extraction by-products, particularly olive pomace. This residue, comprising pulp, stone fragments, and liquid residues, presents a significant untapped resource. While Morocco produces approximately 30,000 tons of olive pomace annually, equivalent to 184 MWh of energy (Najah EL idrissi et al., 2023), the prevailing disposal method involves treating it as waste, leading to adverse environmental impacts (Di Giacomo and Romano, 2022; Ouzounidou et al., 2010). Recent research has unveiled the rich nutritional components within olive pomace, offering opportunities for sustainable alternatives (Albendea et al., 2023; Baker et al., 2023; Dhingra et al., 2012; Li et al., 2022; Lin et al., 2017; Ribeiro et al., 2021). Using olive pomace and wheat straw directly in ruminant feed is problematic due to their poor digestibility. Olive pulp's high lignin content and wheat straw's crystalline cellulose make them challenging for animals to digest. To enhance their nutritional value and reduce waste, processing techniques should be developed to improve digestibility. Using fungi to treat agricultural residues is an eco-friendly method that enhances digestibility (Intasit et al., 2022; W. Sun et al., 2022). Fungi like Fusarium, Penicillium, and Phanerochaete produce enzymes like ligninase, cellulase, and hemicellulose,

which break down tough compounds like lignin and cellulose (benaddou et al., 2023; Gupta and Chundawat, 2020; Méndez-Líter et al., 2021; Z. Sun et al., 2022). Lignin, a sturdy plant material, resists digestion by traditional enzymes (Zhang et al., 2023). Fungal enzymes like lignin peroxidase, laccase, manganese peroxidase, and versatile peroxidase are specialized to break down lignin into simpler forms (Astuti et al., 2022; Bilal et al., 2023; Kumar and Chandra, 2020; Kumar et al., 2022). Similarly, cellulases from fungi efficiently convert cellulose into sugars, making them more digestible for animals (Astuti et al., 2022).

Combining olive pulp with wheat straw and applying fungal treatment offers a promising solution. This study seeks to enhance the nutritional value of olive pulp by combining it with wheat straw, and concurrently investigates the efficacy of fungal treatment employing *Fusarium oxysporum* and *Phanerochaete chrysosporium* fungi. Another primary aim is to discern the superior fungal candidate for treating the blend through a comparative analysis of their performances. The secondary objective revolves around identifying the optimal duration for achieving lignin degradation, cellulose improvement, and enhancement of in vitro true digestibility (IVTD) within fungal-treated substrates.

MATERIAL AND METHODS

Substrate preparation

The chemical analysis of the selected substrates is presented in Table 1. Olive pulp particles ranged in size from 2 to 5 mm, while wheat straw (WS) particles were 0.5 to 1.5 cm in size. The cleaned substrates were soaked in water for 3 days at room temperature, facilitating complete penetration of the water throughout the material(benaddou et al., 2023). After draining excess water (giving a final dry matter content of 420 g/kg for olive pulp and 330 g/kg for WS), five distinct mixing ratios between olive pulp and WS were prepared: 4:4 (100% of OP and 0% of WS (OP)), 3:4(75% of OP and 25% of WS (MOP)), 2:4 (50% of OP and 50% of WS (OPWS), 1:4(25% of OP and 75% of WS (MWS)) and 0:4 (0% of OP and 100% of WS(WS)). These diverse mixing ratios were chosen to cover a broad spectrum of compositions, allowing for a comprehensive exploration of the effects of fungal treatment and substrate composition on digestibility and lignin degradation. For each ratio specified, precisely 10 g of dry matter was weighed and placed in sterile Petri dishes. These materials (together with the petri dishes) were then autoclaved at 121°C for 20 minutes, and stored at room temperature until their intended use.

Table 1. The chemical composition of olive pulp and wheat straw

Component	Olive pulp	Wheat straw
DM (%)	66.74 ±1.20 [a]	84.92 ± 1.00 [c]
OM (%)	98.45 ± 2.00 [a]	90.50 ± 2.10 [b]
Ash (%)	1.30 ± 0.17 [a]	9.30 ± 0.20 [b]
NDF (%)	60.4 ± 0.17 [a]	87.4 ± 2.3 [b]
ADF (%)	48.2 ± 0.17 [a]	69.4 ± 1.3 [b]
ADL (%)	17.81 ± 1.02 [a]	12.12 ± 2.40 [b]
Crud protein (%)	2.30 ± 0.24 [a]	3.40 ± 0.09 [a]
Crude fat (%)	3.9 ± 1.03 [a]	0.9 ± 0.23 [b]
Total sugar (%)	4.10 ± 0.78 [a]	1.2 ± 0.23 [b]
Calcium (g/Kg)	12.10 ± 0.43 [a]	4.23 ± 0.24 [b]
Copper (mg/Kg)	24.78 ± 0.23 [a]	4.17 ± 0.33 [b]

Fungal strains and spawn preparation

We employed two fungi, *Phanerochaete chrysosporium* and *Fusarium oxysporum*, to assess their impact on the nutritional value and environmental sustainability of olive pulp. *Fusarium oxysporum* was selected based on its successful application in enhancing the nutritional value of olive agricultural co-products, specifically olive pomace and wheat straw (Benaddou et al., 2023; M'Barek et al., 2019)). Additionally, *Phanerochaete chrysosporium* was chosen based on prior literature (Z. Sun et al., 2022; Zhang et al., 2012). The initial cultivation of the selected fungi (as shown in Table 2) was carried out using Czapek medium (Li et al., 2023).The preparation of spawn involved introducing fragments of colonized agar culture into sterilized sorghum grains, followed by incubation at 28°C until the mycelium completely colonized all the grains. Colonized sorghum grains were utilized as spawn to inoculate the substrates.

Fungal growth

The estimation of fungal growth was accomplished through the determination of mycelium surface area. This methodology entailed measuring two fundamental dimensions: the maximum

Table 2. Enzyme activity of *P.chrysosporium* and *F. oxysporum*

Description	Enzyme activity	Strains	
		P.chrysosporium	*F. oxysporum*
Cellulase activity (IU.ml^{-1})	Endoglucanase	1.3	0.64 ± 0.1
	β-glucosidase	N.D	0.68 ± 0.1
Ligninase activity (IU.ml^{-1})	Laccase	3.4	1.04 ± 0.1
	Lignin peroxidase	0.5	N.D
	Manganese peroxidase	1.2	N.D

length (D1) and the maximum width (D2) of the mycelium surface. The initial step involved preparing samples, wherein 15g-DM OP and WS were positioned within Petri dishes. Subsequently, the samples were autoclaved (120°C at 20 min) and then inoculated with prepared spawn in the center of petri dishes aseptically condition and subjected to incubation under 28°C. Observation and measurement procedures were conducted using binocular loupes. The measurement of the maximum length (D1) involved identifying the farthest dimension from one end of the mycelium surface to the other. Similarly, the maximum width (D2) was measured by gauging the dimension perpendicular to the length. Utilizing the measurements of D1 and D2, the total mycelium surface area was calculated using the formula:

$$Mycelium\ area = [(D1 + D2)/2]^2 * \pi \quad (1)$$

Subsequent analysis of the obtained results facilitated the evaluation of mycelium growth relative to different assessment durations (0, 6, 12, and 18 days). The positive control was the fungi growth in Czapek medium.

Substrates inoculation

Approximately 1±0.5 g of the prepared spawn (colonized sorghum grains) was be added to 10 g of each prepared substrate. Using sterile spoons and tweezers, the spawn was be mixed aseptically, to be equally distributed through the substrate. The samples (petri dishes with inoculated substrates) were be incubated at 28°C under 70-80% humidity. All treatments were be tested at the same time and was be repeated three times.

Sampling

Every four weeks, from week zero to week twelve, a sample (a petri dishes with a substrate being treated with a used strain) of each ratio was be taken out of the incubator and fungal treatment was be discontinued; and samples was no longer be treated aseptically. Each sample was be observed macroscopically; then mixed by hand. The treated sample was be dried at 60°C until constant weight. The dried sample was ground over a 1 mm sieve for chemical (fibers, proteins, etc.) and biological (digestibility) analyses.

Physical, chemical, and biological analyses

Fiber analysis

Fiber analysis was performed according to the method described by Van Soest et al., (1991). To assess the impact of fungal treatment on lignocellulosic materials, untreated samples of olive pulp (OP) and wheat straw (WS) were included as controls. These controls played a critical role in the calculation of several parameters. Lignin loss (L_loss) is calculated as follows:

$$\text{L\_loss} = \frac{ADL_c - ADL_t}{\text{ADL}_c} \times 100 \quad (2)$$

where: ADL_c is ADL of control (untreated) and ADL_t is ADL of the treated sample after 4, 8 or 12 weeks. The cellulose improvement (C_imp) was calculated by relation follow:

$$\text{C\_imp} = \frac{(ADF_t - ADL_t) - (ADF_c - ADL_c)}{(ADF_c - ADL_c)} \times 100 \quad (3)$$

where: ADF_t and ADF_c represented the ADF values of the treated and control samples (untreated), respectively, after 0, 4, 8, and 12 weeks. ADL_t and ADL_c indicated the ADL values of the treated and untreated samples, respectively, measured during the same time intervals for ADF assessment.

If C_imp was less than 0, it indicated cellulose degradation. Similarly, if L_loss was less than 0, it implied lignin release.

In vitro true digestibility

In vitro true digestibility (IVTD) was be achieved using the ANKOM DAISYII Incubator (Nayan et al., 2019a). The improvement of IVTD (IVTD_imp) was calculated following same the fiber analysis calculation method.

$$\text{IVTD\_imp} = \frac{IVTD_t - IVTD_c}{IVTD_c} \times 100 \qquad (4)$$

where: $IVTD_c$ – *IVTD* of control and $IVTD_t$ is *IVTD* of the treated sample after 4, 8 or 12 weeks.

Crude fat and crude protein determination and mineral composition

We determined crude protein (CP) content according to the Kjeldahl × 6.25 method and crude fat (CF) content using the Soxhlet method (Sharifian Fard et al., 2014). Calcium and Copper were anlysed base on Hitchen and Zechanowitsch method (1980).

Dry mater

For dry matter (DM) determination, air-dried material was dried at 103 °C for 4 h. Ash content was determined by combustion for 3 h at 550°C in a muffle furnace. The data for three replicate samples were averaged and expressed as %.

Enzymatic activity

Laccase activity was determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as substrate, and lignin peroxidase assay was determined using the dye azure B as a substrate (Hermosilla et al., 2018; Srinivasan et al., 1995). MnP activity was measured by monitoring the oxidation of Mn^{2+} to Mn^{3+} in 0.11 M sodium lactate, and the enzyme activities were expressed in U/l. Cellulase activity was determined by Ghose (1987) method.

Data processing and statistical analysis

A multifactor ANOVA analysis was conducted using R software to assess the influence of various factors, including the mixing ratio of wheat straw and olive pulp (OP, MOP, OPWS, MWS, and WS), fungal treatment (*F. oxyporum* and *P. chrysosporium*), treatment duration (0, 4, 8, and 12 weeks), and their interactions, on the studied parameters (L_loss, C_imp, IVTD_imp, and RS) in both fresh and treated substrates (n = 180, including 5 ratios × 3 treatments (2 fungi + control) × 4 durations × 3 replicates). Post-hoc multiple comparisons were performed using Tukey's test at a significance level of $\alpha = 0.05$ to determine significant differences between treatments. Additionally, the relationships between IVTD, cellulose, and lignin were analyzed through correlation analysis using Origin software version 2023 (10.0), with correlations presented as Pearson correlation coefficients (r).

RESULTS

The study examined the effects of fungal treatments, specifically employing *F. oxysporum* and *P. chrysosporium*, on five different mixing ratios of olive pulp (OP) and wheat straw (WS) - OP, MOP, OPWS, MWS, WS - with the aim of enhancing their suitability for ruminant feed. These treatments, conducted at 28°C, successfully reduced lignin content ($p<0.001$) and increased cellulose across all ratios ($p<0.05$). The findings indicated that the OP-to-WS ratio had a significant impact on the digestibility of the treated substrates with both fungi ($p<0.001$).

Comparative growth of *P. chrysosporium* and *F. oxysporum* in different substrates

This study assessed the growth of *P. chrysosporium* and *F. oxysporum* in different substrates. Both fungi exhibited significant growth in the CZAPEK medium ($p<0.001$) and preferred olive pomace (OP) over wheat straw (WS) ($p<0.05$) (Fig. 1). *P. chrysosporium* initiated growth in OP and WS on the second day, but its expansion over 12 weeks was remarkable in OP (110 cm²) compared to limited growth in WS (20 cm²). *F. oxysporum* demonstrated different growth patterns, reaching a mycelium surface of 20 cm² in OP after four days, expanding to 70 cm² in 12 weeks. In WS, growth was less pronounced, with a mycelium surface of 3 cm² in 12 days. These results highlight OP as the preferred growth substrate and variations in growth dynamics between the two fungi.

Impact of fungal treatment and substrate compositions on cellulose content dynamics

This study explored the impact of fungal treatment and substrate compositions on cellulose

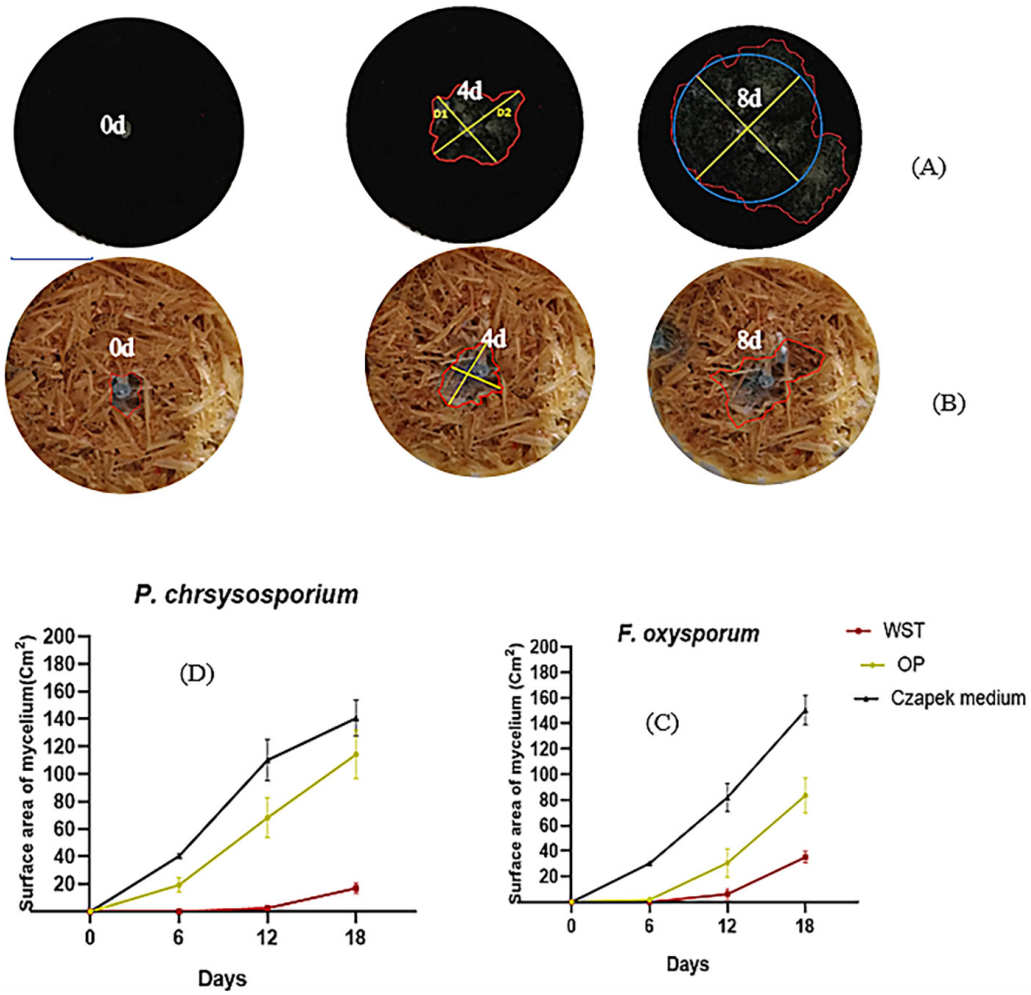

Fig. 1. Estimation of fungal growth based on incubation duration: determination of mycelium surface of *F. oxysporum* treating olive pulp (a) and wheat straw (b). development of mycelium surface of *F. oxysporum* (c) and *P. chrysosporium* (d) in WS, OP, and Czapek Medium over incubation time

content dynamics, measured as mean C_imp values. In the olive pomace (OP) substrate, both *Phanerochaete chrysosporium* and *Fusarium oxysporum* treatment resulted in a decrease in cellulose content after 12 weeks, with mean C_imp values of -8.51 ± 15.03 and -8.51 ± 15.03, respectively. A similar trend was observed in olive pomace with wheat straw (OPWS), where *P. chrysosporium* treatment led to a decrease in cellulose content (mean C_imp was -5.88 ± 7.75). Conversely, in the case of myceliated olive pomace (MOP), both fungal species contributed to an increase in cellulose content after 12 weeks, with a mean C_imp of 13.1 ± 2.3. The study also noted a general pattern of cellulose increase from 4 to 8 weeks, followed by a decrease from 8 to 12 weeks across various substrates. These findings provide valuable insights into the cellulose content dynamics impacted by fungal treatment and substrate composition (Fig. 2).

Fungal-induced lignin degradation across different substrate ratios and treatment duration

This study investigated fungal-induced lignin degradation across various substrate ratios and treatment durations, measured as lignin loss (L_loss). Both *Fusarium oxysporum* and *Phanerochaete chrysosporium* treatments resulted in a significant reduction ($P < 0.001$) in lignin content across all substrate ratios. *P. chrysosporium* consistently exhibited higher mean lignin loss values, ranging from 24.22%±13.75 at 4 weeks to 31.57%±20.65 at 12 weeks, while *F. oxysporum* treatment resulted in mean lignin loss ranging from 19.27%±11.08 at 4 weeks to 23.67% ± 14.11 at 12 weeks. The study revealed notable differences in lignin degradation across substrates. After 12 weeks of treatment, the WS substrate exhibited a lignin loss (L_loss) of 14.46 ± 2.7 for F.

oxysporum and 7.84 ± 3.62 for *P. chrysosporium*. In contrast, the MWS, OPWS, and MOP substrates showed higher lignin loss values, with MWS (25% OP and 75% WS) treated with *P. chrysosporium* exhibiting the highest average of 62%. Lignin degradation increased over time for OP and MWS substrates following fungal treatment. OPWS demonstrated consistently stable lignin degradation, while the WS substrate exhibited a declining trend. Comparing OP and OPWS substrates revealed no significant difference between the two fungi within the same treatment duration. However, variations were observed across different durations. Notably, MWS exhibited higher L_loss values compared to OP. MOP treated with *P. chrysosporium* showed an increase in lignin degradation from 4 weeks to 12 weeks, while *F. oxysporum* exhibited constant lignin degradation levels.

Effect of OP and WS ratio on in vitro true digestibility of fungal-treated substrates

This study examined the influence of the olive pomace (OP) and wheat straw (WS) ratio on the in vitro true digestibility (IVTD) of fungal-treated substrates. The results indicated a significant impact of the OP-to-WS ratio on substrate digestibility with both *Fusarium oxysporum* and *Phanerochaete chrysosporium* treatments ($P < 0.001$). Mixed substrates, including mixed olive pomace (MOP), olive pomace and wheat straw (OPWS), and mixed wheat straw (MWS), demonstrated significantly higher IVTD values compared to non-mixed substrates (OP and WS). The IVTD improvement (IVTD_imp) values after treatment with both fungi were notably higher for MOP (58.56% ± 16%), OPWS (54.18% ± 20%), and MWS (36.83% ± 18%) compared to OP (26.25% ± 11%) and WS (14.43% ± 7.48%). Overall, IVTD consistently increased across all substrates and treatment durations, with a distinct rise in IVTD values from the 4-week mark to the 12-week mark. Notably, both OPWS and MOP substrates consistently displayed higher IVTD values compared to other substrates throughout all durations. While the IVTD of WS treated with both fungi remained relatively lower during the 4-week treatment period, it gradually increased over time.

Cluster and correlation

The cluster diagram resulting from Principal Component Analysis (PCA) (Fig. 3) showed that OPWS and MOP shared a large area between them, while MWS shared a small area with the other two (OPWS and MOP). This demonstrated that OPWS and MOP were not significantly different. MWS was positively correlated with IVTD (Fig. 3(A) and Fig. 3(B)). In Figure 4, the classification of substrates treated by the two fungi at different durations is shown. The substrates that align most effectively with our objectives are characterized by a substantial degradation of lignin (high L_loss), a significant increase in digestibility (high IVTD_imp), and the simultaneous preservation of cellulose (C-imp >= 0). As depicted in Figure 3, the mixed substrates (MWS, OPWS, and MWS) demonstrate the most favorable response to our objectives. Notably, MWS and OPWS, when treated with *P. chrysosporium*, and OPWS and MOP, when treated with F. *oxysporum*, exhibit the most promising outcomes in line with our objectives. This implies that the most advantageous ratios, considering the drilling aspect, involve a blend of 50% OP and 50% WS, as well as a combination of 25% OP and 75% WS straw.

DISCUSSION

The study's results align with the significance of substrate mixing ratios in feedstock modification. The varying ratios of OP and WS create diverse compositions that affected the accessibility of fungal enzymes to lignin and cellulose.

Influence of fungal treatments and substrate compositions on cellulose content and lignin degradation in lignocellulosic materials

The outcomes of the study indicated that the impact of fungal treatments on cellulose content and lignin degradation was influenced by specific substrate compositions and the types of fungi used ($p < 0.001$). Fungi have the ability to use both lignin and cellulose as carbon sources, but their preferences may vary depending on various factors. In general, fungi tend to favor cellulose as a more easily accessible carbon source (van Kuijk et al., 2015a). It's important to note that cellulose, a major component of lignocellulosic materials, played a crucial role in determining the digestibility and nutritional value of the feedstock (Chen et al., 2024).

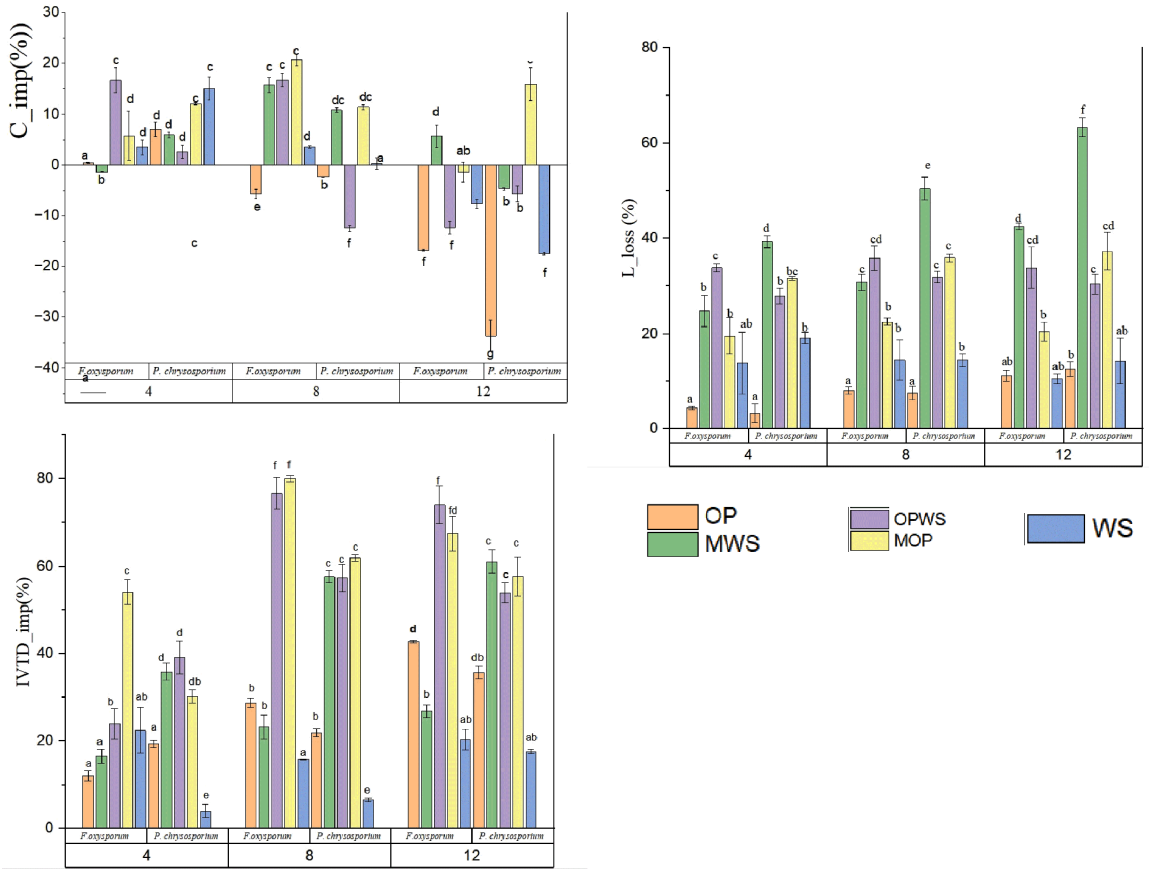

Fig. 2. Effect of fungal treatment on cellulose improvement (C_imp), lignin loss (L_loss) and IVTD improvement (IVTD_imp) in different substrate ratios: OP (100% OP, 0% WS), MOP (75% OP, 25% WS), OPWS (50% OP, 50% WS), MWS (25% OP, 75% WS), WS (0% OP, 100% WS)

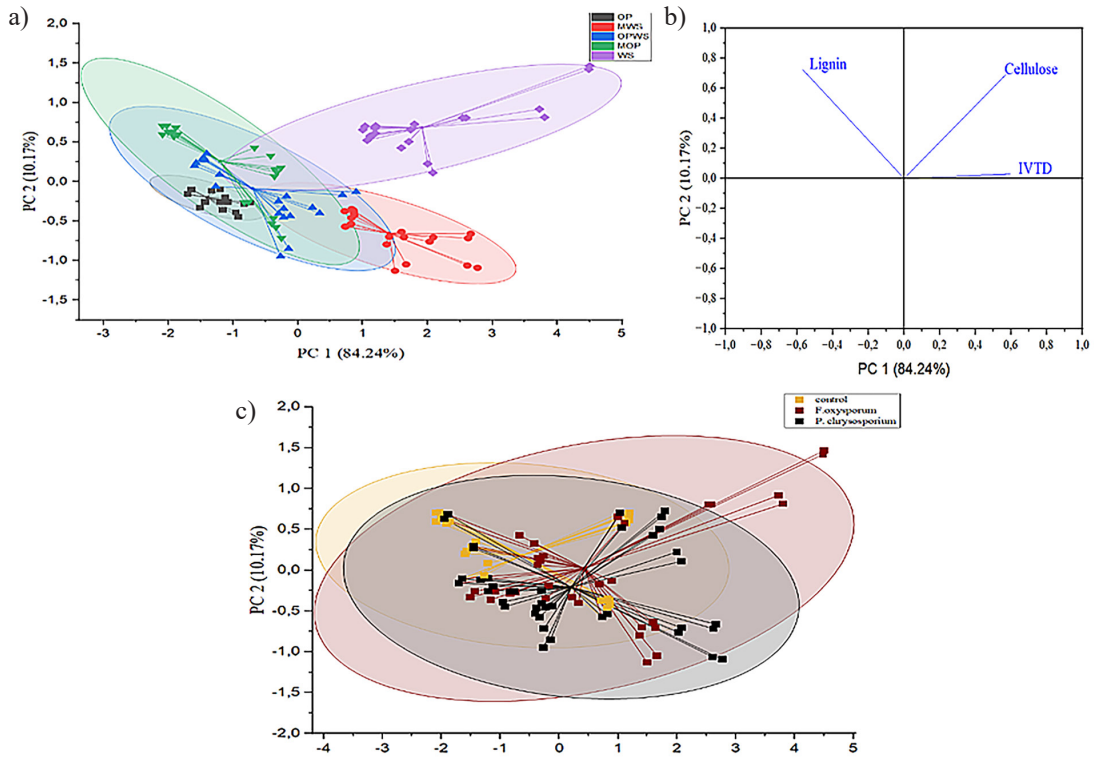

Fig. 3. Principal component analysis (PCA) (A), cluster plot from PCA (B) and substrate clustering (C)

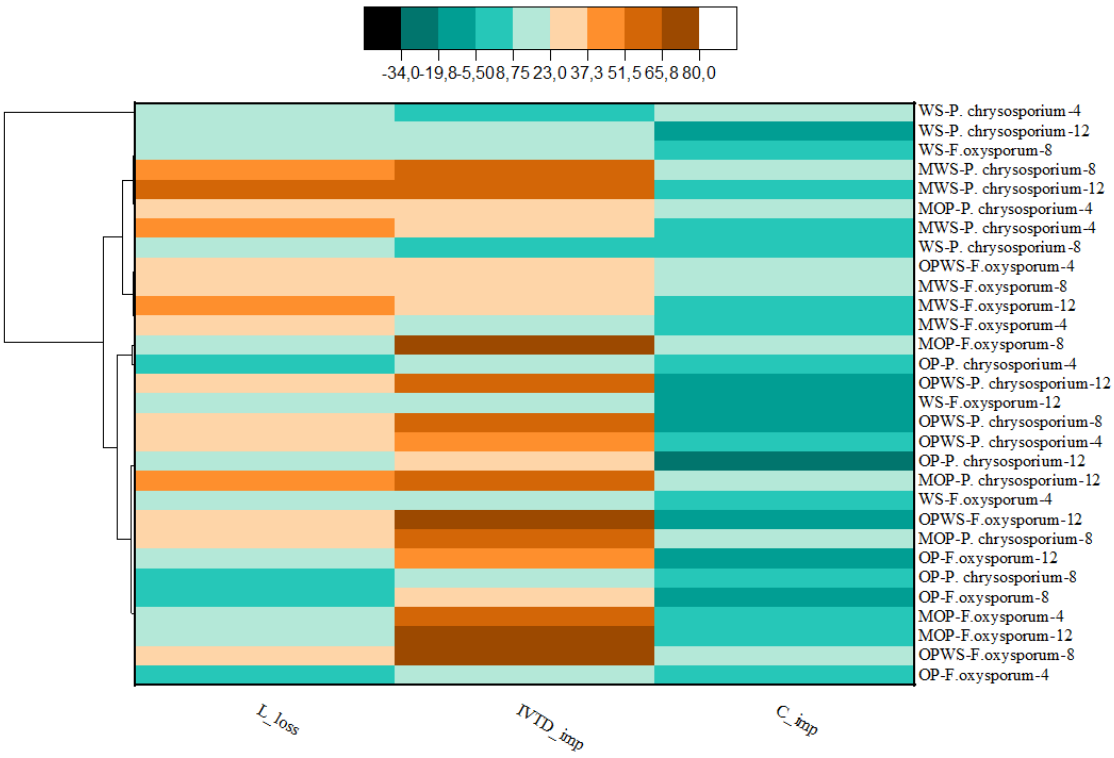

Fig. 4. Heatmap clustering of treated substrate ratios with *F. oxysporium* and *P. chrysosporium* over treatment duration (e.g., OP-F.*oxysporum*-4 represents treated olive pomace with *F. oxysporium* for 4 weeks)

The observed decline in cellulose content, as case of OP (100% OP and 0% WS) and treated OPWS with *P. chrysosporium* could indeed be attributed to the enzymatic degradation of cellulose by fungi. *P. chrysosporium and F. oxysporum* produced Endoglucanase and β-glucosidase that specifically target and break down cellulose into smaller sugar molecules to use it as carbon source (Table 1). After fungi degrade cellulose, it is often observed that they do not degrade lignin as efficiently, as the case of OP. This difference in cellulose and lignin degradation is attributed to the complex and resistant characteristics of lignin, as well as the metabolic strategies of fungi (Mendu et al., 2022). Both fungi were able to partially hydrolyze degraded cellulose into simple sugars, which explained the improved digestibility of the organic matter (OP) after their treatment.

The inclusion of wheat straw into the composition of olive pulp (OP) at a ratio of 3/4, as observed in the case of MOP (comprising 75% OP and 25% WS), or at a ratio of 1/4, as seen in the case of MWS (comprising 25% OP and 75% WS), revealed an intriguing outcome concerning fungal behavior. Notably, this outcome highlighted the ability to retain cellulose while simultaneously degrading lignin with both *P. chrysosporium* and F. *oxysporum*. Consequently, the addition of 25% WS exhibited a beneficial effect on the drilling aspect, presenting a positive result that was in line with the objectives of our study. Indeed, several studies have demonstrated that as lignin content decreases, as case of MOP and MWS, it can lead to increased digestibility due to improved access of digestive enzymes to other components like cellulose and hemicellulose (Hu et al., 2023; van Kuijk et al., 2015b).

In the cases of OPWS (50% OP and 50% WS) and WS (0% OP and 100% WS), both *P. chrysosporium* and *F. oxysporum* exhibited the remarkable capability to simultaneously degrade cellulose and lignin. It's worth noting that the cellulose degradation was relatively low when OPWS was treated with F. *oxysporum*. This observation suggested that the introduction of 50% WS to OP activated fungal mechanisms that had developed complex enzymatic systems. These systems encompassed cellulases and lignin-degrading enzymes, such as peroxidases and laccases. By collaborating in a coordinated manner, these enzymes effectively disassembled the intricate lignocellulosic structure (Chandel et al., 2020). However, this outcome was not in line with the objective of achieving a favorable borehole, as it involved cellulose degradation alongside lignin degradation.

Synergistic effects of mixed substrate ratios on fungal degradation of lignocellulosic materials

The improvement in digestibility and lignin loss observed in the mixed substrate ratios (MWS, MOP, and OPWS) compared to the non-mixed substrates (WS and OP) can be attributed to the synergistic effects of combining olive pulp (OP) and wheat straw (WS) in different ratios. When these substrates are mixed, they create a more balanced composition that allows for better interaction between the enzymes (such as peroxidases, laccases, and cellulases) produced by the fungal treatments (*F. oxysporum* and *P. chrysosporium*) and the lignin and cellulose components (Table 2) (Ouzounidou et al., 2010). As shown in Table 1, OP and WS each bring distinct biochemical compositions to the substrate matrix. OP was characterized by a high lignin content, which hampers its biodegradability and which is known to be a recalcitrant compound that hinders feedstock digestibility and nutrient availability (Bentil, 2021; Zwane et al., 2019). On the other hand, WS consisted of a higher cellulose content and lower lignin content. When these two materials are combined, the resulting mixture exhibited a diverse spectrum of lignocellulosic compounds that can engage a wider range of fungal enzymes (Hu et al., 2023).

Moreover, the presence of both OP and WS in the mixed substrates contributes to a diverse range of nutrients, structural components, and chemical properties. This diverse composition enhances the accessibility of fungal enzymes to lignin, facilitating a more efficient breakdown of lignin and consequently leading to higher lignin loss (Saini and Sharma, 2022). In the context of successful fungal treatment of lignocellulosic materials, proper colonization of the substrate by mycelium is crucial. This colonization is necessary for the effective degradation and transformation of the material. During the treatment process, the greater fungal growth was observed in olive pulp (OP) compared to wheat straw (WS). The mean of growth rate of two fungi in OP was 5.56 ± 1.44 Cm^2/day but in WS was 0.95 ± 0.48 Cm^2/day (Fig. 1(A)). Mycelium extend and grow into the substrate, secreting enzymes as they go (Chai et al., 2022; Xia et al., 2022). This process allows the mycelium to penetrate and explore the substrate, accessing nutrients and breaking down organic matter. The difference in the growth rate in OP and WS could be attributed to the favorable nutritional composition of OP (Pritsch and Garbaye, 2011). As shown Table 1, OP is characterized by higher levels of crude fatty acids and other nutrients, including mineral salts (calcium, copper, and manganes). These components serve as readily available sources of carbon and minerals, which in turn promote rapid fungal colonization and enzymatic activity. Moreover, Olive pulp has a significantly higher total sugar content compared to wheat straw. Sugars are readily available carbon sources for fungi, and the elevated sugar content in olive pulp could support faster fungal growth (Albendea et al., 2023; Innangi et al., 2017; Lammi et al., 2019; Leite et al., 2016; Najah El Idrissi et al., 2023; Ouzounidou et al., 2010; Paz et al., 2020; Weinberg et al., 2008). In contrast, non-mixed substrates (WS and OP) may lack the optimal balance of nutrients and components required for effective fungal activity. The absence of complementary components might hinder the efficiency of the fungal treatments, resulting in less lignin degradation and potentially lower digestibility (Anyango, 2023).

Optimal duration for lignin degradation, cellulose improvement, and IVTD enhancement in fungal-treated substrate

The duration needed for fungal treatment is another crucial aspect (Sandra J.A. van Kuijk et al., 2015). In this study presented here, fungal treatment was conducted over varying time periods (4, 8 and 12 weeks) to assess whether the degradation of lignin transitions to cellulose degradation with extended treatment. Determining the optimal treatment duration is crucial for maximizing the breakdown of lignin while preserving cellulose and hemicellulose (Tsegaye et al., 2020). If the treatment is too short, lignin degradation might remain incomplete, limiting the accessibility of cellulose for subsequent enzymatic digestion (Nayan et al., 2019b). Conversely, excessively long treatment might lead to excessive cellulose degradation, reducing the overall nutritional value of the treated material. Understanding the timing of the transition from lignin degradation to cellulose degradation can provide valuable insights into the effectiveness of different fungal species and substrate ratios in terms of digestibility (Tsegaye et al., 2020).

In our investigation into the effects of fungal treatments on various substrates, our findings align and expand upon existing literature. When we consider the optimal treatment durations for maximizing key parameters, we can draw valuable

comparisons with prior studies. Specifically, in the context of in vitro true digestibility (IVTD), our results corroborate previous research by demonstrating that extending the treatment duration to approximately 12 weeks consistently yields the most effective outcomes across all substrates. This extended duration is in line with findings from studies emphasizing the importance of longer treatment times for achieving substantial improvements in IVTD values (Agrawal et al., 2011; van Kuijk et al., 2017). However, regarding cellulose content, our study concurs with earlier research that suggests the conservation and improvement of cellulose content exhibit their best results within a shorter timeframe of around 4 to 8 weeks, which aligns with the general trend of increased cellulose content from 4 weeks to 8 weeks, followed by a decrease from 8 to 12 weeks (Zheng et al., 2023). Moreover, our investigation highlights the role of an extended treatment period of about 12 weeks in achieving the most substantial reductions in lignin content. This finding resonates with prior studies that have underscored the importance of longer durations for effective lignin degradation (Chen et al., 2023). Collectively, our study both reinforces and extends the existing literature by providing specific timeframes tailored to different substrates and fungal species, offering practical guidelines for feed optimization and aligning with the broader scientific consensus in this field.

For the OP substrate, treatment with both *P. chrysosporium* and F. *oxysporum* resulted in significant lignin degradation across all durations, which aligns with previous findings (Akyol et al., 2019). However, it's noteworthy that the optimal treatment duration for lignin degradation differed between the two fungi. In our study, *P. chrysosporium* consistently exhibited higher lignin loss values, with an average of 24.22%±13.75 at 4 weeks, increasing to 31.57% ± 20.65 at 12 weeks. Conversely, F. *oxysporum* treatment showed comparatively lower lignin loss values, ranging from 19.27% ± 11.08 at 4 weeks to 23.67% ± 14.11 at 12 weeks. These results offer a nuanced perspective compared to the literature, suggesting that the choice of fungal species plays a crucial role in determining the optimal duration for lignin degradation. Moreover, cellulose improvement was observed within the first 8 weeks of treatment in our study, followed by a decline at the 12-week mark. This observation is consistent with prior studies emphasizing the importance of early treatment stages for cellulose preservation (Zheng et al., 2023). Additionally, in vitro true digestibility (IVTD) consistently increased across all treatment durations compared to the untreated control, with a significant rise from the 4-week mark to the 12-week mark. Taking these findings into account and referring to Fig. 3 and Fig. 4, our study suggests that an optimal treatment duration of 8 weeks was identified for enhancing the nutritional value of OP substrate when treated with both *P. chrysosporium* and F. *oxysporum*, providing practical insights into the influence of fungal selection and treatment duration on lignocellulosic material optimization. In the case of OPWS, both fungi initially increased cellulose content over the initial 4 weeks; however, this cellulose improvement was followed by subsequent degradation. Notably, lignin degradation remained relatively stable across the different durations, suggesting a consistent impact. IVTD values exhibited a notable improvement from the 4-week mark to the 12-week mark, with both fungi causing degradation at the 12-week point. Interestingly, *F. oxysporum* induced a more significant degradation in IVTD compared to *P. chrysosporium*. As a result, the optimal treatment duration for OPWS with both fungi appears to lie between 4 and 8 weeks, with the most favorable outcome potentially leaning towards the 4-week mark.

For the MOP substrate, both *P. chrysosporium* and *F. oxysporum* exhibited an enhancement in cellulose content over the 4 and 8-week durations, followed by a slight decrease at 12 weeks, particularly noticeable for F. *oxysporum*. Intriguingly, lignin degradation displayed an increasing trend over the treatment duration, with *P. chrysosporium* resulting in an L_loss of 36% at 12 weeks, while *F. oxysporum* exhibited an L_loss of 20%. Remarkably, IVTD values consistently improved, culminating in an impressive IVTD_imp of 58.56% ± 16% at the 12-week mark. In conclusion, a comprehensive strategy could involve an initial 8-week treatment to enhance cellulose content, followed by an extended 12-week treatment to achieve substantial lignin degradation and maximize IVTD for the MOP substrate. In the case of MWS, *P. chrysosporium* significantly increased cellulose content during the 4-week period, and *F. oxysporum* exhibited no impact. F. *oxysporum*, however, significantly increased MWS cellulose (C_imp) more than *P. chrysosporium* during the 8-week duration. Interestingly, lignin degradation consistently increased over the treatment duration for both fungi. IVTD_imp of MWS

treated with *P. chrysosporium* consistently exceeded that of *F. oxysporum* across all durations. Taking all of these factors into account, it appears that for the MWS substrate, an optimal treatment duration of around 8 weeks could be advantageous. During this period, both fungi demonstrated significant cellulose improvement and substantial lignin degradation. Additionally, the consistently higher IVTD_imp values associated with *P. chrysosporium* treatment suggest that the 8-week duration could help achieve a balance between maximizing cellulose improvement and enhancing digestibility.

For WS, *P. chrysosporium* induced an increase in cellulose over 4 weeks, while *F. oxysporum* showed no impact. After 12 weeks, *F. oxysporum* sustained the cellulose increase (C_imp), while *P. chrysosporium* showed no effect. Notably, both fungi caused cellulose degradation after 12 weeks, with *P. chrysosporium* inducing a more substantial degradation (C_imp) compared to F. *oxysporum*. Lignin degradation exhibited a minor discrepancy between the two fungi over the three durations, with WS demonstrating a declining trend. Remarkably, IVTD consistently improved, showing a gradual increase over the durations. Considering these factors, an optimal treatment duration for maximizing lignin degradation could be within the early durations, around 4 to 8 weeks. This is supported by the observed trend of lignin reduction and early cellulose improvement induced by *P. chrysosporium*. For maximizing cellulose improvement, a treatment duration of around 4 weeks might be effective due to *P. chrysosporium*'s cellulose-enhancing effect within this timeframe. For IVTD enhancement, extending the treatment duration to around 12 weeks could be beneficial, considering the consistent improvement observed over this timeframe.

CONCLUSIONS

In conclusion, our study delved into the intricate interplay among fungal treatments, substrate compositions, and the specific fungal types employed, namely *Phanerochaete chrysosporium* and *Fusarium oxysporum*, to shed light on their substantial impact on cellulose content and lignin degradation. These fungi demonstrated a clear preference for cellulose as a carbon source, leading to its degradation. Importantly, the addition of wheat straw in carefully selected ratios emerged as a promising technique to strike a balance between cellulose retention and lignin degradation. Fascinatingly, certain scenarios showcased the simultaneous degradation of both cellulose and lignin, driven by intricate enzymatic systems. These discoveries underscore the complex dynamics that unfold between fungi, substrate compositions, and the unique attributes of lignocellulosic materials. In summary, the innovative aspect of our work lies in uncovering the synergistic effects achieved by blending olive pulp (OP) and wheat straw (WS) in varying ratios. This novel approach resulted in heightened digestibility and enhanced lignin loss within the mixed substrate combinations (MWS, MOP, and OPWS), a distinct improvement over the non-mixed substrates (WS and OP). The key behind this synergy lies in the harmonious composition of mixed substrates, which facilitates intensive interactions between fungal enzymes and lignocellulosic components. This, in turn, translates to the breakdown of lignin and heightened enzymatic access.

The study further revealed the substantial impact of substrate composition on fungal growth and colonization. Notably, OP exhibited higher fungal colonization due to its favorable nutritional profile, bolstering enzymatic activity and growth. The elevated sugar content within OP acted as a catalyst for accelerated fungal growth, creating a conducive environment for efficient enzymatic activity. Conversely, non-mixed substrates lacked these synergistic components, potentially hindering fungal efficiency. Our results underscore the pivotal role of substrate composition in influencing the efficacy of fungal degradation. Additionally, we unveiled that the ideal treatment duration for enhancing in vitro digestibility consistently spanned 12 weeks across various substrates. However, the specific substrate type played a pivotal role in determining the optimal treatment duration. In essence, shorter durations of 4 to 8 weeks were found to be most effective for cellulose improvement, while the 12-week timeframe proved optimal for substantial lignin degradation. This nuanced effect showcases the importance of tailoring strategies to match the unique nutritional goals of each substrate. Ultimately, our study offers novel insights that hold the potential to revolutionize feed value optimization and sustainability in the realm of livestock production.

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