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Eco-Friendly Utilisation of Agricultural Coproducts: Enhancing Ruminant Feed Digestibility through Synergistic Fungal Co-Inoculation with *Fusarium solani*, *Fusarium oxysporum*, and *Penicillium chrysogenum*

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ABSTRACT

This study aims to explore the synergistic effects of co-inoculation with *Fusarium solani* (F.s), *Fusarium oxysporum* (F.o), and *Penicillium chrysogenum* (P.ch) to enhance the digestibility and quality of lignocellulosic biomass for ruminant feeding. Wheat straw (WS), olive pomace (OP), and cedar wood (CW) were assessed as substrates. Results indicated varying impacts on lignin loss (L_loss), cellulose improvement (C_imp), and *in vitro* true digestibility improvement (IVTD_imp). F.o and P.ch co-inoculation exhibited the highest mean L_loss (53.74%), surpassing F.s and P.ch co-inoculation (18.23%) and F.s and F.o co-inoculation (19.23%). F.o_P.ch co-inoculation notably increased cellulose content (C_imp = 29.86 ± 18.19%) and IVTD_imp (40.74% ± 20.51%), while F.o_F.s showed minimal IVTD_imp (0.14 ± 11.42%). Substrates differed in fiber change and dry matter loss, with OP having the highest C_imp (25.6 ± 20.7%). Treatment duration influenced L_loss and IVTD_imp, increasing from 4 to 12 weeks. Co-inoculating F.o and P.ch enhances lignin degradation and biomass digestibility, improving their suitability for ruminant feed. Thoughtful selection of fungal combinations is crucial for optimizing co-inoculation. These findings support the utilisation of lignocellulosic biomass in ruminant feed.

Keywords: fungal treatment, lignin loss, cellulose improvement, digestibility improvement, co-inoculation, lignocellulosic biomass.

INTRODUCTION

The competition for food resources between animals and humans necessitates the exploration of alternative strategies to ensure sustainable food production. Lignocellulosic biomass, a highly abundant and underutilized resource, holds great potential for addressing the challenges in ruminant animal feed production (Padam et al., 2014; Ravindran and Jaiswal, 2016). In Morocco, a country with a significant agricultural and food industry, substantial amounts of agro-industrial waste, including wheat straw, wood sawdust, and olive pomace, are generated annually (Adusei-Gyamfi et al., 2022; Agyeman and Duah Lin, 2022; Paz et al., 2020).

A significant amount of agro-industrial waste remains unutilized, either through incineration or burial. This is primarily due to its structural characteristics that impede direct industrial utilization, leading to detrimental environmental consequences, such as air pollution and energy loss (Bavaro et al., 2017). However, this waste primarily consists of biodegradable lignocellulosic biomass (Cheng and Whang, 2022). The valorisation of this biomass as a feed source presents an intriguing alternative to its disposal, particularly in regions where these by-products

are generated in substantial quantities within short timeframes (Bernal et al., 2016). Consequently, it is regarded as one of the most cost-effective sources of carbohydrates and holds great potential as a substrate for producing various high-value products, including biofuels, as well as for ruminant nutrition (Den et al., 2018; Van Kuijk et al., 2015; Van Kuijk et al., 2017a, 2016a, 2016b; Vázquez-Vuelvas et al., 2021).

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Lignocellulosic biomass typically is high in cellulose (25–53%), hemicelluloses (20–35%), and lignin (10–25%), forming a tightly intermeshed structure held together by both covalent and non-covalent bonds(Rouches et al., 2019; Sajid et al., 2022) Among these components, cellulose, which is the most abundant carbohydrate globally with an annual production exceeding 7.5×10^{10} tons, and hemicellulose, are enzymatically converted into fermentable sugars and serve as crucial dietary sources for ruminant animals (Van Kuijk et al., 2016b, 2016c). However, the nutritional utilization of lignocellulosic biomass is hindered by the presence of lignin, which is the most challenging chemical constituent to extract from lignocellulosic biomass (Brinchi et al., 2013). Lignin acts as an inhibitor of the enzymatic process, reducing sugar yields (Isikgor and Becer, 2015). Furthermore, lignin is a water-insoluble and heterogeneous biopolymer composed of cross-linked monolignols, including coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S) (Patil et al., 2016).

To raise the accessibility of cellulose and hemicellulose, and thus their degradability, several biological, physical, and/or chemical methods have been developed selectively to remove or modify lignin before feeding lignocellulosic biomass to ruminants (Diouri and Wiedmeier, 2000; Guo et al., 2022; Kalčíková et al., 2014). Among biological treatment agents, fungi are more oxidatively powerful than bacteria (Brown and Chang, 2014; Nayan et al., 2020). Fungal treatment is also inexpensive and environmentally friendly biotechnology (Kalčíková et al., 2014; Pant and Kuila, 2022; Shirkavand et al., 2016; Van Kuijk et al., 2016b). That has gained attention as an alternative to other treatments for animal nutrition (Van Kuijk et al., 2017b). The performance of fungi in the treatment of lignocellulosic biomass comes from their ability to secrete extracellular enzymes capable of degrading lignin and facilitating the access of rumen microbes to structural carbohydrates (Duskaev et al., 2021; Rouches et al., 2019). In addition to lignin content and composition, which are the main factors of low lignocellulosic biomass digestibility in ruminants, there are other factors, such as cellulose crystallinity, which have been reported as negative factors in biomass enzymatic digestibility (Hermosilla et al., 2018; Wu et al., 2013).

In recent years, co-inoculation, the simultaneous introduction of multiple fungal strains, has emerged as a potential strategy to enhance the degradation of lignocellulosic biomass. Co-inoculation offers the advantage of combining different fungal strains with complementary enzymatic activities, potentially resulting in synergistic effects on fiber degradation and biomass quality improvement. The simultaneous action of multiple strains can enhance enzymatic activity, broaden substrate utilization, and lead to novel cooperative interactions that further enhance lignocellulosic biomass degradation.

The objective of this study was to investigate the effectiveness of co-inoculation in improving the digestibility and quality of lignocellulosic substrates, considering varying durations of treatment. In addition to analysing the degradation of cellulose and lignin, both individually and through co-inoculation with *P. chrysogenum, F. solani, and F. oxysporum*, we introduced sampling intervals at 4, 8, and 12 weeks to comprehensively track the temporal progression. By evaluating indicators like dry matter loss and fiber loss during these intervals, we gained a comprehensive understanding of temporal trends. Furthermore, we delved into potential synergistic effects within this time-bound framework, adding depth to our observations. Our investigation also addressed the influence of treatment duration, aiming to uncover whether extended exposure triggers a shift from lignin degradation towards cellulose degradation.

MATERIAL AND METHODS

Fungal strains and culture

The selected fungal strains, including *P. chrysogenum, F. solani, and F. oxysporum,* were obtained from culture collections. The initial cultures of the fungal strains were grown on Czapek medium at 25°C.

Substrate preparation

Three different lignocellulosic substrates were selected: wheat straw (WS), cedar sawdust (CS), and olive pomace (OP). WS was obtained from fields in the region of El Hajeb, Morocco. OP came from Olea Food company in Meknes, Morocco. CS was sourced from Ifrane National Park, Morocco, chosen for its high lignin content and cellulose crystallinity.

WS and CS were chopped into particles measuring 0.5 to 1.5 cm in size, while OP particles had a size of 2 to 5 mm. The chopped substrates were soaked in water and left to fully penetrate the material for 3 days at room temperature. Excess water was then drained off for 6 hours. The substrates were autoclaved at 121°C for 20 minutes to ensure sterilization. Approximately 30 g of dry matter substrate was weighed into 250 ml glass bottles, covered with cotton to allow air exchange, and sealed. The containers with sterilized substrates were autoclaved again at 121°C for 20 minutes and stored at room temperature (25 ± 3°C) until use.

Spawn preparation

The initial culture of the selected fungi was carried out in Czapek's medium. To prepare the spawn, sterilized sorghum grains were inoculated with a 10 mm disc of colonized agar culture. The inoculated sorghum grains were then incubated at 25°C for 15 days until fully colonized grains were obtained.

Substrate inoculation and co-inoculation

For single fungal treatments, 4.5 ± 0.5 g of the prepared spawn (colonized sorghum grains) was added to each 30 g (DM) substrate. The spawn and substrate were accurately weighed. Using sterile spoons and tweezers, the spawn was mixed aseptically to ensure equal distribution throughout the substrate. The samples (250 ml glass bottles with inoculated substrates) were incubated at 26°C under a controlled relative humidity of 70–80%.

For co-inoculation treatments, the following combinations were prepared: F. solani + *F. oxysporum* , solani + P. chrysogenum, and *P. chrysogenum* + *F. oxysporum* For each combination, 2.25 ± 0.25 g of each spawn was mixed and added to the 30 g (DM) substrate, resulting in a total of 4.5 ± 0.5 g of spawn per substrate. The co-inoculated samples were incubated under the same conditions as the single fungal treatments.

Sampling

Samples were collected at 4, 8, and 12 weeks from the beginning of the trial. Each sample consisted of a 250 ml glass bottle containing one substrate treated with one fungal strain or a co-inoculation combination. The samples were taken out of the incubator, and fungal treatment was discontinued. Each sample was mixed by hand and then dried at 60°C for 72 hours.

Dry matter loss

The dry matter loss (DM_{loss}) was calculated as the difference in dry matter content between the initial sample and the respective time point using the formula (1):

$$DM_{loss} = \frac{DM_i - DM_t}{DM_t} \times 100 \quad (1)$$

where: DM_{loss}: Dry matter loss (%),
DM_i: Dry matter content at the beginning of the trial (%),
DM_t: Dry matter content at the respective time point (%) (treated Dry matter).

Physical and chemical analyses

Fiber analysis

Fiber analysis was performed using the method of Van Soest, Robertson, and Lewis. In this study, the term 'fiber' refers to the combined content of cellulose and lignin. The lignin content was defined as acid detergent lignin (ADL), and cellulose was calculated as the difference between acid detergent fiber (ADF) and ADL.

To assess the loss of cellulose (2) and lignin (2) during the fungal treatment, the percentage of fiber loss was calculated using the following formula:

$$C\_imp = (C_{timpoint} - C_i)/C_i) \cdot 100) \quad (2)$$

$$L\_loss = (L_i - L_{timpoint})/L_i) \cdot 100) \quad (3)$$

where: *C_imp* and *L_loss*: The percentage of cellulose improvement and lignin loss
C_i or L: cellulose or lignin content at the beginning of the trial
$C_{timpoint}$ and $L_{timepoint}$: cellulose and lignin content at the respective time point (4w, 8w, or 12w).

A positive percentage indicates a decrease in cellulose and lignin content. Conversely, a negative percentage indicates an increase in cellulose and lignin content.

In vitro true digestibility

In vitro, true digestibility (IVTD) was measured using the ANKOM DAISYII Incubator (Gulecyuz, 2017). The improvement in in vitro true digestibility ($IVTD_{imp}$) was calculated using the following formula:

$$IVTD_{imp} = \\ = (IVTD_i - IVTD_{timpoint})/IVTD_i) \cdot 100) \quad (4)$$

where: $IVTD_{imp}$: The percentage of improvement in in vitro true digestibility
$IVTD_i$: IVTD at the beginning of the trial
$IVTD_{timpoint}$: IVTD at the respective time point (4w, 8w or 12w).

Enzymatic Activity

After drying under 60°C for 72 hours, whole maize was milled and passed through a 1 mm sieve. Enzymatic extraction was carried out as described by Rodrigues et al. (2008) with some modifications (Ghose, 1987). Laccase, lignin peroxidase, and manganese peroxidase activities were measured using specific substrates and following established protocols. Cellulase activity was determined using the GHOSE method (Ghose, 1987).

Statistical analysis

All experiments were conducted in triplicate. The obtained data were analyzed using analysis of variance (ANOVA) in the R software. The sources of variation taken into consideration were fungal species, substrates, blocks (representing treatment periods), and treatment duration. To assess data normality, the Shapiro-Wilk test was employed, while homoscedasticity was checked using the Bartlett test. In cases where the conditions for ANOVA were not met, the Kruskal-Wallis test was utilized. Multiple comparisons were performed using Tukey's test to determine significant differences between treatments.

RESULTS AND DISCUSSION

Characteristics of the strains and the substrates

The physicochemical characteristics of the substrates were presented in Table 1. We note that the three substrates are approximately equally rich in total fibers but different in lignin content. Table 2 shows that, regardless of the selection medium, all the fungi have both cellulase and ligninase activities.

Means ((±SD) indicated by the same letter are not different significantly according to Tow-way ANOVA at α = 0,05. DM: Dry Matter (just before treatment), OM: Organic matter, C: Cellulose, L: lignin

Means ((±SD); ND: non-detected; CM: culture medium in which the cellulose was the only source; LM: culture medium in which the lignin is the only source of carbon; LCM: culture medium in which both cellulose and lignin are the sources of carbon.

Effect of fungal treatment

The analysis of variance (ANOVA) revealed significant effects of the factors and interactions on the response variable. Fungi had a highly significant effect ($p < 0.0001$). The interaction between 'fungi' and 'substrate' ($p < 0.0001$), as well as the interactions between 'fungi' and 'treatment

Table 1. Chemical and physical composition of the lignocellulosic materials

Chemical composition	Olive pomace	Cedar sawdust	Wheat straw
DM (%)	66.74 ±1.20 [a]	59.41 ± 1.90 [b]	79.22 ± 2.00 [c]
OM (%)	98.45 ± 2.00 [a]	98.40 ± 1.40 [a]	90.50 ± 2.10 [b]
Ash (%)	1.30 ± 0.17 [a]	1.10 ± 0.10 [a]	9.30 ± 0.20 [b]
C (%)	33.17 ± 2.10 [a]	43.98 ± 1.90 [b]	42.23 ± 1.89 [b]
L (%)	20.18 ± 1.02 [a]	18.75 ± 0.10 [a]	12.12 ± 2.40 [b]
IVTD (%)	42.1 ± 1.02 [a]	34.7 ± 0.5 [b]	58.84 ± 1.2 [c]
Crude protein	2.30 ± 0.24 [a]	3.20 ± 0.21 [a]	3.40 ± 0.09 [a]

Table 2. Enzymes activities measured after a 10-days of incubation in submerged fermentation

Enzyme activity	Enzyme type	*P. chrysogenum*	*F. solani*	*F. oxysporum*
Cellulase activity ($IU \cdot ml^{-1}$)	Endoglucanase	N.D	0.64 ± 0.1	N.D
	β-glucosidase	0.68 ± 0.1	1.05 ± 0.1	2.93 ± 0.2
Ligninase activity ($IU \cdot ml^{-1}$)	Laccase	2.3 ± 0.1	1.04 ± 0.1	N.D
	Lignin peroxidase	N.D	N.D	6.47 ± 0.1
	Manganese peroxidase	3,3 ± 0.1	N.D	N.D

duration' ($p < 0.0001$), all showed highly significant effects. This showed that a significant proportion of the variability in the response variable could be explained by the combined influence of the factors.

Fungal species showed variations in their impact on L_loss (Fig. 1A and Table 3). In terms of individual inoculations, the results demonstrated that F.o had a mean lignin loss value of 36.58%, whereas P.ch exhibited a lower value of 15.166%. These findings are consistent with previous studies conducted by Rodriguez et al. (2006), who reported lignin loss levels of 15.5% and 15.4% for F.o and P.ch, respectively(Rodriguez et al., 2006).

The previous study explored the impact of co-inoculation. Notably, F.o_P.ch displayed the highest mean L_loss value of 53.74%, surpassing both F.s_P.ch and F.o_F.s, which showed L_loss values of 18.23% and 19.23%, respectively. Therefore, despite P.ch individually showing lower levels of lignin degradation, the addition of F.o resulted in a synergistic effect, leading to a more efficient breakdown of lignin. It is plausible that F.o and P.ch produce distinct lignin-degrading enzymes that work synergistically when combined. For instance. the presence of specific enzymes such as lignin peroxidase from F.o and laccase from P.ch may act cooperatively to break down lignin (Dashtban et al., 2010; Rodriguez et al., 1994) (Table 2). The study showed varying effects on cellulose loss with different fungal treatments (Fig. 1B) and Table 3). Cellulose is a complex carbohydrate found in plant cell walls and represents a major source of energy for ruminants Specificall like *Lentinula edodes* (Van Kuijk et al., 2015a) . An increase in cellulose content was observed in the F.o treatment (C_imp = 23.86 ± 18.87%), F.o_F.s treatment (C_imp = 13.29 ± 10.94%), F.s treatment (C_imp = 16.49 ± 9.68%), and F.s_P.ch treatment (C_imp = 11.70 ± 29.86%). The decrease in cellulose content was observed in the P.ch treatment. Notably, the F.o_P.ch treatment (C_imp = 29.86 ± 18.19%) showed a significant increase in cellulose content.

Fusarium oxysporum and Penicillium chrysogenum, when combined, likely exhibited lignin-degrading capabilities. Lignin, being a complex polymer, can be a barrier to accessing cellulose(Beltrán-Flores et al., 2023). However, the synergistic action of F.o and P.ch resulted in the breakdown of lignin, which exposed and preserved the cellulose. As shown in Figure 1 (C) and Table 3, among the individual fungal treatments. *F. oxysporum* (F.o) demonstrated the highest improvement in in vitro true digestibility (IVTD) with a mean value of 40.70±14.34%. Similarly, the co-inoculation treatment of *F. oxysporum* and *P. chrysogenum* (F.o_P.ch) showed a significant IVTD improvement with a mean value of 40.74±20.51% which can be explained by enzymatic synergy. In contrast, the combination treatment of *F. oxysporum* and F. solani (F.o_F.s) showed a minimal IVTD improvement of -0.14 ±11.42%). This result can be attributed to the

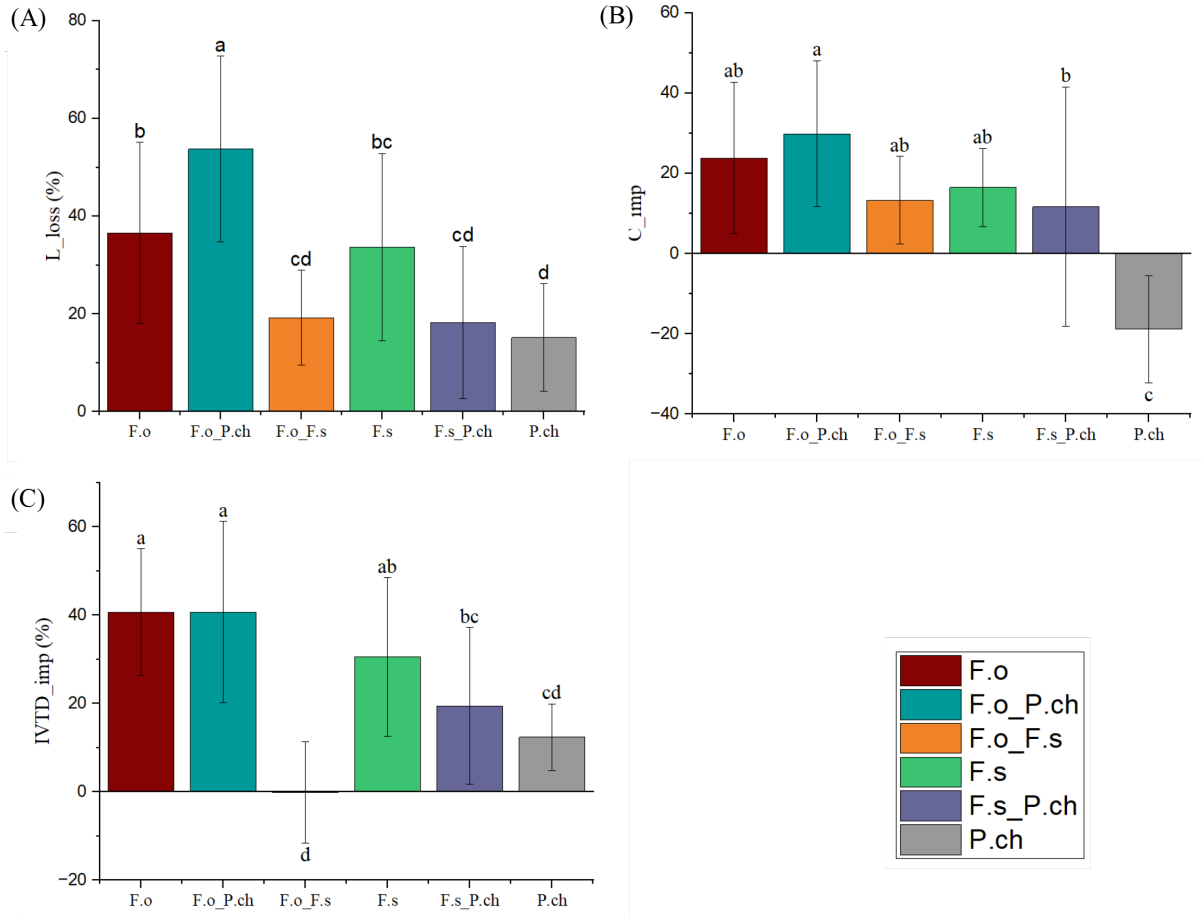

Fig. 1. Lignin loss (L_loss), Cellulose loss (C_imp) and IVTD loss (IVTD_loss) of all treated substrate with different fungi: F.o – *Fusarium oxysporum;* P.ch – *Penicillium chrysogenum;* F.s_P.ch – *Fusarium solani* + *Penicillium chrysogenum* (co-inoculation*);* F.s – *Fusarium solani;* F.o_P.ch – *Fusarium oxysporum* + *Penicillium chrysogenum* (co-inoculation)

potential production of compounds or enzymes by some fungal species that inhibit or interfere with the activity of other fungi. This interference can hurt the breakdown of complex carbohydrates, such as cellulose or hemicellulose, which ultimately results in decreased digestibility(Ejechi and Obuekwe, 1994). In the present study, the effects of different fungal treatments on dry matter loss (DM_loss) were evaluated (Fig. 3). DM included components such as carbohydrates (e.g. cellulose), proteins, fats, minerals, and lignin(Lee et al., 2023). The treatment with *F. oxysporum* (F.o) and with co-inoculation of *F. oxysporum* and *P. chrysogenum* (F.o_P.ch) resulted in a moderate level of substrate degradation (mean DM_loss was 14.74±7.80% and 14.43±6.30%) respectively). In contrast, the co-inoculation treatment of *F. oxysporum* and *F. solani* (F.o_F.s) showed d significantly higher mean DM_loss. All the fungi degraded the dry matter to nourish themselves, but this degradation should not have been excessive for the fungal treatment to be effective. According to the results, the combination of F.o and P.ch proved to be the best compared to other combinations as it preserved the dry matter, especially cellulose, and increased digestibility.

Effect of substrate

The study investigated the effects of co-inoculation on the digestibility and biodegradability of lignocellulosic substrates. As shown in Figure 2 and Table 3, there is a significant difference between substrate on fiber change (lignin loss and cellulose loss) and DM loss. The assessment of dry matter loss (DM_loss) in different substrates unveiled distinct manifestations of the substrates' impact on the degradation of lignocellulosic materials (Fig. 3). Among the substrates. WS demonstrated the highest mean DM loss of 20.39%, signifying a substantial reduction in dry matter content.

Table 3. Chemical composition and digestibility of substrates treated with single and co-inoculation

Fungus	Duration	Weat straw			Cedar wood			Olive pomace		
		ADL	Cellulose	IVTD	ADL	Cellulose	IVTD	ADL	Cellulose	IVTD
F. oxysporum	0w	11.04±1.2	50.75±2.4	58.92±1.5	19.68±0.71	36.9±0.71	34.8±0.28	21.1±1.29	34.44±1.79	42.04±2.37
	4w	8.83±0.49	56.7±0.81	83.73±2.58	17.35±0.13	36.55±0.68	41.22±0.5	18.27±0.47	39.14±0.15	53.18±3.94
	8w	5.76±0.29	68.88±0.05	87.75±0.08	9.71±0.01	43.53±0.13	46.91±0.32	10.38±0.11	47.71±1.11	66.72±0.4
	12w	3.97±0.06	80.46±0.58	91.56±0.4	9.45±0.17	38.27±0.71	50.29±0.28	7.29±0.01	45.1±0.38	68.87±1.18
F. oxysporum + P. chrysogenum	0w	11.04±1.2	50.75±2.4	58.92±1.5	19.68±0.71	36.9±0.71	34.8±0.28	21.1±1.29	34.44±1.79	42.04±2.37
	4w	6.35±0.64	61.32±1.39	61.3±1.41	15.25±0.07	38.85±0.78	45.37±0.04	14.3±0	40.3±0	55.3±4.5
	8w	4.14±0.23	69.27±1.46	73±0.77	7.8±0.71	41.3±1.41	50.49±0.26	7.6±0.42	44.35±1.34	68.8±0.71
	12w	2.35±0.78	83.82±2.13	83.3±4.24	5.85±0.07	43.7±0.42	58.85±0.78	4.7±0.28	50.3±0	73.45±0.07
F. oxysporum + F. solani	0w	11.04±1.2	50.75±2.4	58.92±1.5	19.68±0.71	36.9±0.71	34.8±0.28	21.1±1.29	34.44±1.79	42.04±2.37
	4w	8.32±0.03	46.81±2.11	54.43±1.7	17.3±0	37.8±0.71	31.4±0	18.3±0	38.35±0.07	41.3±1.41
	8w	7.3±0	57.71±6.49	55.54±0.34	15.95±0.49	38.8±2.12	42.65±0.49	15.35±0.07	39.86±0.79	41.38±1.52
	12w	7.05±0.1	61.16±1.19	56.15±0.21	12.9±0.71	43.2±2.55	42.85±0.78	14.3±0	42.93±0.81	40.1±0.42
F. solani	0w	11.04±1.2	50.75±2.4	58.92±1.5	19.68±0.71	36.9±0.71	34.8±0.28	21.1±1.29	34.44±1.79	42.04±2.37
	4w	9.19±0.48	54.43±1.7	77.29±2.23	15.47±0.12	37.39±0.87	36.7±0.19	20.07±0.55	38.45±0.15	46.73±0.73
	8w	6.4±0.1	58.71±0.41	89.04±0.56	13.23±0.29	38.72±0.14	41.34±0.73	8±0.11	42.13±0.1	57.56±0.01
	12w	4.43±0	60.86±0.58	96.04±0.73	12.4±0.21	43.77±0.36	43.9±0.15	7.07±0.09	44.49±0	61.18±0.33
F. solani + P. chrysogenum	0w	11.04±1.2	50.75±2.4	58.92±1.5	19.68±0.71	36.9±0.71	34.8±0.28	21.1±1.29	34.44±1.79	42.04±2.37
	4w	11.35±0.07	43.53±0.04	55.9±0.57	17.8±0.71	27.35±1.34	40.8±0.71	18.4±0.14	42.85±0.78	51.66±1.9
	8w	8.35±0.07	45.45±0.13	60.35±0.07	15.45±0.21	34.81±0.69	43.7±0.85	12.3±0	51.3±1.41	55.4±0.57
	12w	8.45±0.21	54.31±5.67	58.8±0.71	14.55±0.35	38.83±0.53	48.31±0.01	8.32±0.03	54.2±1.13	65.55±0.35
P. chrysogenum	0w	11.04±1.2	50.75±2.4	58.92±1.5	19.68±0.71	36.9±0.71	34.8±0.28	21.1±1.29	34.44±1.79	42.04±2.37
	4w	8.48±0.79	38.31±0.98	61.36±2.07	18.05±0.07	26.18±1.2	37.88±0.07	20.81±0.67	32.63±0.4	48.48±2.85
	8w	7.19±0.1	35.26±0.03	74.39±0.15	16.25±0.06	31.1±0.59	35.84±0.25	15.57±0.06	31.43±0.27	49.25±0.16
	12w	8.07±0.11	28.83±0.27	69.06±0.29	13.36±0.44	35.73±0.48	42.52±0.04	14.85±0.06	29.58±0.07	47.23±0.1

OP displayed a marginally lower mean DM_ loss of 18.85% compared to WS, whereas CW showcased the lowest mean DM_loss of 13.6%. Moreover, the analysis revealed that OP showed the lowest mean lignin loss, followed by CW. while WS demonstrated the highest mean lignin loss. That can be explained by propriety characteristics (Table 1). As observed (Fig. 4), there was a positive correlation between lignin content and lignin loss. This correlation has been noted in several studies. highlighting its significance(Van Kuijk et al., 2015b; Zhang et al., 2022) Moreover, the results indicated that different substrates showed varying degrees of cellulose loss or improvement. Among the substrates. OP showed the highest cellulose improvement with a mean cellulose loss (C_imp) of 25.6 ± 20.7%, followed by WS with a C_imp of 10.57 ± 28.05%. On the other hand. CW showed a slight increase in cellulose content with a C_imp of 3.13 ± 13.56%. Fungal cellulases, such as endoglucanases, Exoglucanases, and β-glucosidases. play a crucial role in breaking down cellulose into smaller sugar units that fungi can utilise(Yang et al., 2023). However, these enzymes can face challenges in accessing cellulose when the substrate has a high lignin content, such as in the case of CW and OP.

Regarding the improvement in digestibility (IVTD_imp) compared to the control, the results demonstrated that there were significant differences between the substrates treated with single or co-inoculation methods. Co-inoculation with F.o and Pch had a positive effect on in vitro true digestibility (IVTD_imp) of CW and OP. but it negatively affected the IVTD_imp of WS. The co-inoculation with F.o and P.ch (F.o_P.ch) hurt the IVTD_imp of all substrates. Therefore, while the introduction of multiple fungal strains together in co-inoculation can often result in synergistic interactions and enhanced digestibility, it is not always the case for every fungal combination. In the specific case of F.o and P.ch. the co-inoculation did not lead to the expected positive effects on IVTD improvement. The outcome of co-inoculation can depend on various factors, including the specific fungal strains used, their compatibility, and the characteristics of the biomass substrate.

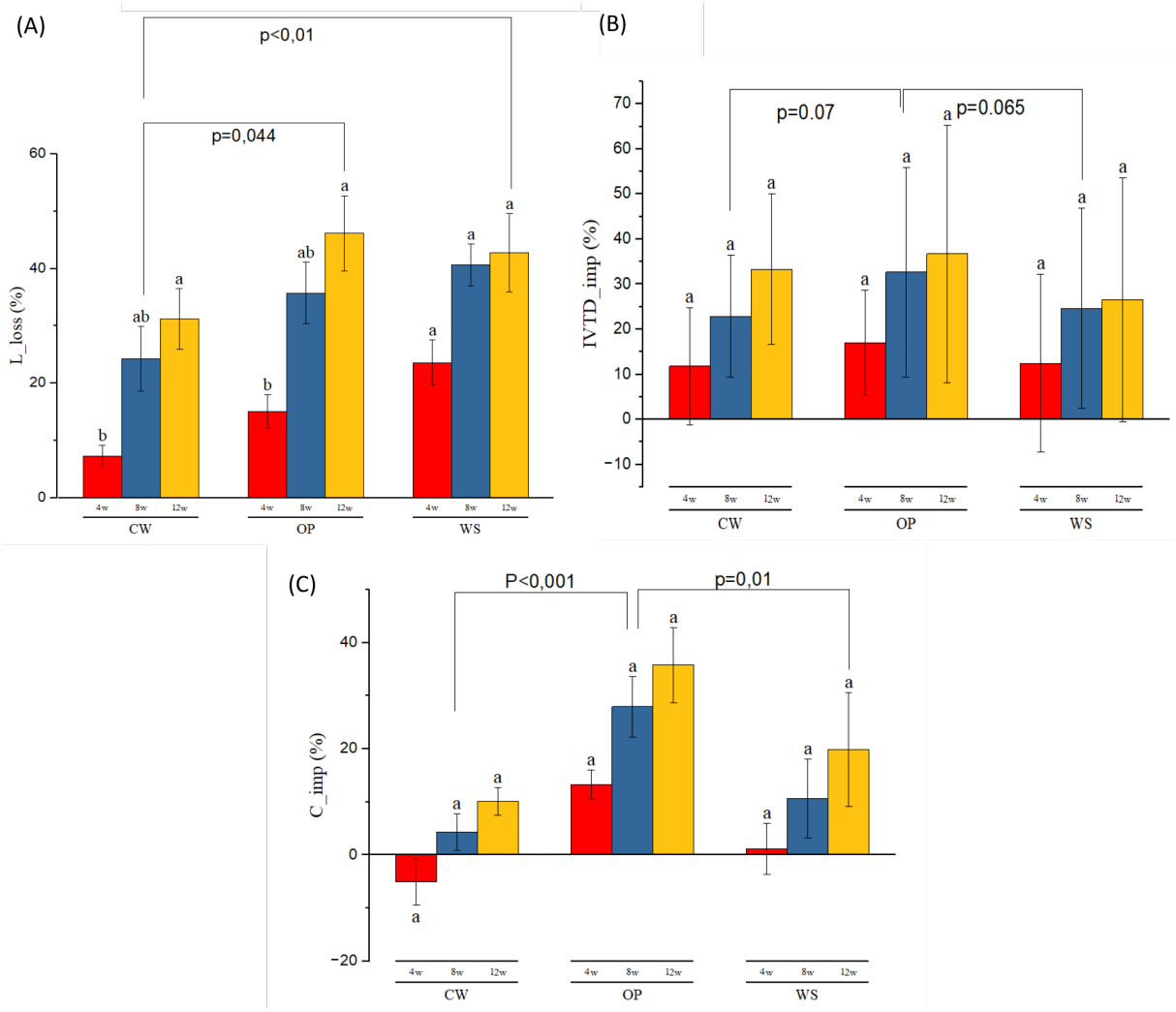

Fig. 2. Lignin loss (L_loss) (A), Cellulose improvement (C_imp) (B) and IVTD improvement (IVTD_imp) (C) of cedar wood (CW), olive pomace (OP), and wheat straw (WS) treated with all fungi during 4, 8 and 12 weeks

Effect of the treatment duration

The results of the study indicate that the duration of treatment had a significant effect on lignin loss, cellulose loss. DM loss and IVTD improvement in the lignocellulosic substrates and fungal treatment.

Indeed, the degradation of lignin, the increase in cellulose, and the digestibility are generally positively correlated with time during fungal treatment of lignocellulosic biomass. At the 4-week duration, the mean lignin loss was 16.09 ± 12.11%. However, at the 8-week duration, there was a substantial increase in lignin loss, with a mean value of 34.89 ± 17.76%. Interestingly, the mean lignin loss at the 12-week duration (36.68 ± 23.39%) was slightly higher compared to the 8-week duration but not significantly different. At the 4-week duration, the mean IVTD improvement was 13.55±15.39 %. However, at the 8-week duration, there was a substantial increase in IVTD improvement, with a mean value of 26.91% ±20.62%. Similarly, at the 12-week duration, the mean IVTD improvement further increased to 31.00% ±23.36%.

The results of the study demonstrate that treatment duration has a significant impact on the dry matter loss (DM_loss) (Fig. 3) of the lignocellulosic substrates. There is a clear upward trend in DM_loss as the treatment duration increases from 4 weeks to 8 weeks and 12 weeks. After 4 weeks of treatment, the mean DM_loss was determined to be 10.83±5.92%. Notably, the DM_loss increased substantially at the 8-week duration, reaching a mean value of 16.73% ±11.29%. The highest level of DM_loss was observed at the 12-week duration, with a mean value of 24.15% ±9.12%.

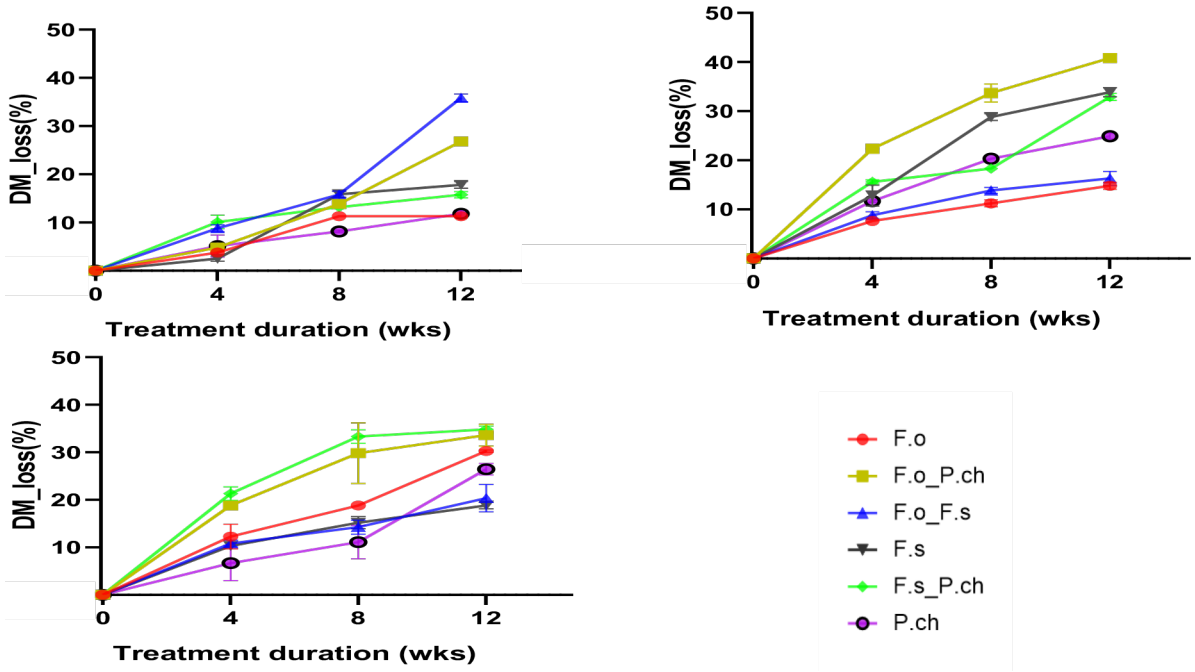

Fig. 3. Effects of co-inoculation with three fungi on dry matter loss of substrate over 4, 8, and 12 weeks

Correlation analysis and principal component analysis

In Figures 5A and 5B was shown that the first two dimensions of analysis expressed 75.98% of the total dataset inertia; indicating that 75.98% of the total variability of the individuals (or variables) cloud was explained by the plane. This high percentage implies that the first plane played a significant role in representing an important part of the data variability. Moreover, the value obtained was considerably greater than the reference value of 39.56%. The classification performed on the individuals revealed the presence of three distinct clusters. Cluster 1(treated CW with P.ch during 4W) was characterised by high values for the variables lignin and cellulose loss. Conversely, this cluster exhibited low values for the variables cellulose, lignin_loss, IVTD, DM_loss, and IVTD_imp, Cluster 2 (samples treated with F.o_F.s and F.s_pch) demonstrated high values for the variables DM_loss and cellulose_loss. On the other hand, this cluster showed low values for the variable IVTD. Cluster 3 (treated WS and OP with F.o_P.ch and F.s and F.o during 8w and 12 w) displayed high values for the variables lignin_loss. IVTD_imp. IVTD, and cellulose. Conversely, this cluster exhibited low values for the variables lignin and cellulose loss.

The correlation table revealed significant relationships among the variables. A strong positive correlation was found between lignin_loss and IVTD_imp, as well as between cellulose and IVTD (r = 0.71 and r = 0.73. respectively). This has been confirmed by several studies (Martens et al., 2023; Van Kuijk et al., 2015; Wan and Li, 2012). On the other hand, a strong negative correlation was observed between cellulose and lignin, cellulose_loss and lignin_loss, and IVTD and lignin (r = -0.75. r = -0.68. and r = -0.78. respectively). Furthermore, the variables DM_loss and lignin_loss showed a weak positive correlation, with a coefficient of 0.24. Additionally, a weak negative correlation was observed between DM_loss and lignin, as well as between DM_loss and cellulose_loss (r = -0.33 and r = -0.28. respectively). Consequently, based on the current study, a positive correlation between digestibility and lignin loss suggests that as more lignin is lost during the treatment process, the digestibility of the biomass increases. This indicates that the breakdown of lignin contributes to the improved accessibility and availability of cellulose and hemicellulose, the carbohydrate components of the biomass, which can be more easily utilized and converted into fermentables.

CONCLUSIONS

In conclusion, our study advances the field through its unique focus on the co-inoculation of

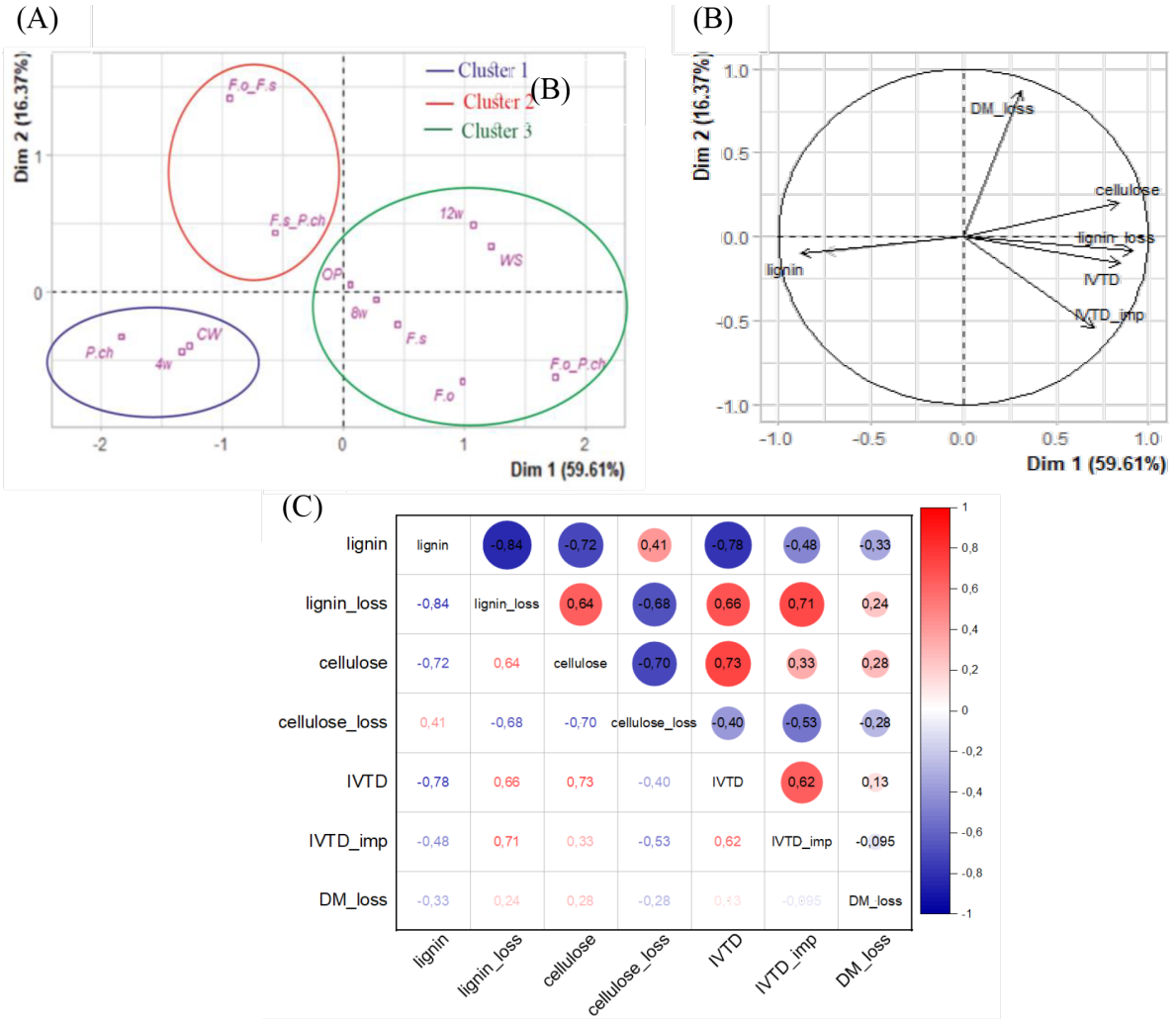

Fig. 4. Correlation of variables and principal component analysis map: exploring interrelationships. (A): Variables factor map (PCA); (B): Qualitative factor map (PCA) and Ascending Hierarchical Classification from the individuals.

F.o – *Fusarium oxysporum;* P.ch – *Penicillium chrysogenum;* F.s_P.ch – *Fusarium solani* + *Penicillium chrysogenum* (co-inoculation); F.s – *Fusarium solani;* F.o_P.ch – *Fusarium oxysporum* + *Penicillium chrysogenum* (co-inoculation)

three distinct *fungi–Fusarium oxysporum, Penicillium chrysogenum,* and *Fusarium solani*–for the treatment of olive pomace, cedar wood, and wheat straw. The pioneering nature of our investigation lies in its revelation of the positive impact of *Fusarium oxysporum* and *Penicillium chrysogenum* co-inoculation on lignin degradation, accompanied by a notable increase in biomass digestibility. This outcome underscores the potential of our approach to elevate the utilization of these lignocellulosic substrates, marking a novel contribution not yet explored by prior researchers. Our study illuminates the transformative potential of co-inoculation in significantly enhancing the efficiency and efficacy of lignocellulosic biomass treatment. This approach unlocks avenues for augmenting lignin degradation, promoting cellulose utilization, and elevating overall digestibility. The exploitation of synergistic interplays among diverse fungal strains within co-inoculation not only charts a promising trajectory for advancing lignocellulosic biomass applications but also holds implications for critical sectors such as biofuel production, biorefineries, and sustainable agriculture. It is important to note, however, that our findings unveiled contrasting outcomes–co-inoculation of *Fusarium solani* and *Fusarium oxysporum*, as well as the *Penicillium chrysogenum* and *Fusarium solani* combination, detrimentally impacted lignocellulosic biomass digestibility while also leading to heightened dry matter degradation. These intricate variations

underscore the necessity of judiciously selecting and evaluating fungal combinations, underscoring the pivotal role of meticulous pairing in securing favorable outcomes concerning digestibility and biomass degradation.

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