Research Article

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Potential use of cowpea (*Vigna unguiculata* (L.) Walp.) stover treated with white-rot fungi as rabbit feed

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Abstract

BACKGROUND: Lignin inhibitory effects within the cell wall structure constitute a serious drawback in maximizing the utilization of fibrous feedstuffs in animal feeding. Therefore treatments that promote efficient delignification of these materials must be applied. This study evaluated the potential of white-rot fungi to upgrade the nutritive value of cowpea stover for rabbit feeding.

RESULTS: There was an increase in the crude protein content of all substrates as a result of fungi treatments, reaching a net gain of 13% for *Pleurotus citrinopileatus* incubation. Overall, net losses of dry and organic matter occurred during fungi treatments. Although the fiber content remained identical, higher consumption of cell wall contents was measured for *P. citrinopileatus* incubation (between 40 and 45%). The incubation period did not influence lignin degradation for any of the fungi treatments. Differences within the fungal degradation mechanisms indicate that *P. citrinopileatus* treatment was most effective, enhancing *in vitro* organic matter digestibility by around 30% compared with the control.

CONCLUSION: Treatment of cowpea stover with *P. citrinopileatus* led to an efficient delignification process which resulted in higher *in vitro* organic matter digestibility, showing its potential in the nutritional valorization of this feedstuff.

Keywords: cowpea stover; white-rot fungi; nutritional valorization; rabbit feeding

INTRODUCTION

Global demand for food sources has been constantly increasing and must be met using fewer available natural resources. This scenario has led to the implementation of policies that enhance the production of legumes for human food within European agriculture. Cowpea is one of the most productive heat-adapted cultivated legumes showing high nutritive value[1] and has been given increased importance recently owing to its adaptability to different environmental conditions. Therefore global intensification of its production is expected.

Besides the agronomic, environmental and economic advantages of including legumes in cropping systems, the production of grains will also generate large amounts of stovers that may have a negative environmental impact if not properly discarded. One of the possible alternatives for the utilization of these disposable resources is their valorization as animal feed. In fact, in extensive Mediterranean production systems, fibrous feeds such as straws and stovers are considered a valuable feed resource in certain periods of the year.[2] Nevertheless, as with many lignocellulosic biomass materials, lignin inhibitory effects within the cell wall structure constitute a serious drawback in maximizing their utilization.[3] Therefore treatments that promote efficient delignification of these materials must be applied. The colonization of fibrous feedstuffs with fungi, more specifically the white-rot wood basidiomycetes, has been studied for some time,[4,5] and recent results have pointed out its efficiency in depolymerizing lignin.[6–9] However, the complexity and specificity of the ligninolytic enzyme systems involved in lignin degradation mechanisms promote variable responses in terms of fungi colonization efficiency. Furthermore, in a recent review, van Kuijk *et al.*[9] have pointed out that the combined effect of fungi on delignification and digestibility is mostly dependent on the fungal strain, the substrate and the extent of fungal biodegradation. Within the substrate level, one must highlight that most studies reporting on the effects of fungi treatments have been conducted using wheat straw. As

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there are substantial differences between grasses and legumes in lignin structure[10] and in linkages between lignin and other cell wall components,[11] mechanisms of fungal degradation should also be different.

Therefore this study aimed to compare the effectiveness of treatments with five white-rot fungal species (*Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus citrinopileatus*, *Pleurotus eryngii* and *Phlebia rufa*), using two incubation periods, on the chemical composition and *in vitro* digestibility of cowpea stover for rabbit nutrition and feeding.

MATERIALS AND METHODS

Fungal species and spawn preparation

The five fungal species used, *G. lucidum* (UF20707), *L. edodes* (UF21403), *P. citrinopileatus* (UF21401), *P. eryngii* (UF21402) and *P. rufa* (156), were preserved in the culture collections of the Laboratory of Mycology and Soil Microbiology and the Laboratory of Biochemistry of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal. Cultivation and spawn preparation were carried out as previously described.[12] To prepare the fungal inoculants, the fungi were transferred to potato dextrose agar plates and incubated at 28 °C until the mycelia colonized most of the plate surface. Subsequently, the colonization of the spawn was carried on wheat grain. Briefly, the wheat grain was hydrated in water and drained. Next, the grain was placed in glass flasks and sterilized in an autoclave at 121 °C for 30 min. After cooling, each flask was inoculated with a 3 cm diameter agar disk containing mycelium and incubated at 25 °C in full darkness for 3 weeks.

Preparation and fungi cultivation on substrate

Cowpea (*Vigna unguiculata*) stover was collected after harvesting of cowpea grains in the south of Spain. The cowpea stover was chopped into lengths of 1–2 cm, then water was added to approximately three times the weight of the stover and left overnight for water absorption by the inner structures. Approximately 50 g portions of cowpea stover were weighed into 250 mL Erlenmeyer flasks and autoclaved at 121 °C for 30 min. In a laminar flow chamber, 2 g of previously prepared wheat spawn was inoculated into each Erlenmeyer flask. The inoculated stover was incubated in triplicate along with the control (autoclaved stover with 2 g of non-inoculated wheat grain) at 28 °C and 75% relative humidity for 22 and 45 days in an incubation chamber (Conviron CMP 3244, Controlled Environments Ltd, Winnipeg, Canada).

Chemical analysis and *in vitro* digestibility

To determine the dry matter (DM) content, samples were dried to constant weight in an air-forced oven at 50 °C and ground over a 1 mm screen (Tecator Cyclotec 1093 Sample Mill, FOSS, Hillerød, Denmark). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) fractions were determined by detergent methodologies without the use of sodium sulfite.[13,14] The concentration of hemicellulose was calculated as the difference between NDF and ADF, and that of cellulose as the difference between ADF and ADL.

Dried samples were analyzed for ash (no. 942.05) and total N as Kjeldahl N (no. 954.01) according to AOAC[15] methods. The crude protein (CP) content was calculated as N × 6.25. Following a three-step methodology, the *in vitro* digestibility (IVD) of organic matter (OM) in rabbits was determined using several enzymes that simulate the digestibility process in the stomach, small intestine and caecum.[16]

Losses in the chemical composition following fungal incubation were calculated as the difference between the control and the inoculated substrate.

Statistical analysis

Data were analyzed with the GLM procedure of SAS[17] as a completely randomized design experiment using one-way analysis of variance (ANOVA), considering fungal treatments (performed in triplicate) as the main effect. When the *F* test was significant ($P < 0.05$), multiple comparisons among means were examined by the Tukey test. All chemical and *in vitro* digestibility analyses were performed in triplicate.

RESULTS AND DISCUSSION

The autoclaving process resulted in a substrate (control) with higher ($P < 0.05$) ADL content and decreased ($P < 0.05$) IVOMD (Table 1). The application of pressurized steam to fibrous feeds promotes changes in the cell wall architecture, depolymerizing lignin and partially hydrolyzing cellulose and hemicellulose molecules.[18] However, this process is dependent on the types of lignocellulosic materials and on the pressure and temperature conditions of the autoclave.[19,20] Therefore, although a decrease in cell wall components is normally reported for substrates treated with pressurized steam,[21] the data we have obtained may result from DM losses associated with steam treatment of substrates.[22] In fact, sugars and other soluble components may volatilize, leading to what may only represent a change in proportion of other compounds such as lignin. Nevertheless, we should also point out that polymerization reactions between hydrolyzed components and lignin may also occur during the application of pressurized steam,[23] contributing to an increase in its concentration. Furthermore, the decrease in IVOMD indicates that changes have occurred in the substrate during the sterilization process using autoclaving, suggesting possible modifications in the polymer structure limiting its accessibility to enzymes. Taking into account that lignin composition and existing linkages between it and carbohydrates may modify the efficiency of fungal colonization,[9] possible alternative methodologies such as chemical sterilization, already reported by Pandey *et al.*,[24] need to be evaluated.

With the exception of *P. eryngii* (22 days of incubation), an increase ($P < 0.05$) in the CP content (Table 1) was measured for all substrates at the end of both incubation periods. Although initial studies[25,26] have pointed out that fungi could fix atmospheric nitrogen, earlier data reported by Millbank[27] indicated exactly the opposite. However, this issue was quite controversial, because the evidence supporting this capability was not completely irrefutable. Furthermore, data published by Kurtzman[28] raise the possibility that increases in the nitrogen content of incubated substrates might be due to the presence of nitrogen-fixing bacteria, a suggestion also supported by Pandey *et al.*[24] More recently, Walker and White[29] considered fungi to be non-diazotrophic (cannot fix nitrogen) and need to be supplied with nitrogen-containing compounds. Thus, although increased protein content during fungal incubation has been reported elsewhere,[30,31] one must be judicious in its interpretation. Another suggestion might be that, while the total Kjeldahl N method does not quantify nitrates and nitrites,[32] some white-rot fungi species possess the ability to use

Table 1. Chemical composition (g kg^{-1} DM) and *in vitro* digestibility (g kg^{-1} OM) of cowpea stover autoclaved, intact and incubated with different fungi for 22 and 45 days

Fungus/sample	Incubation	CP	NDF	ADF	ADL	HC	Cellulose	Ash	IVDOM
Ganoderma lucidum	22	168.3bcd	618.6 cd	454.6 cd	125.9de	164.1a	328.6bc	100.6f	283.4b
Lentinula edodes	22	163.0b	670.0ef	493.4e	112.4bc	176.5a	381.1e	86.0ab	372.3cde
Pleurotus citrinopileatus	22	207.5f	472.5a	347.5a	81.5a	125.0a	266.0a	122.1 h	465.5 h
Pleurotus eryngii	22	149.7a	618.3 cd	464.8de	116.6bcd	153.5a	348.2 cde	89.4bc	369.2 cd
Phlebia rufa	22	173.6cde	593.0c	426.1bc	121.0cde	166.9a	305.1ab	97.8ef	381.6def
Ganoderma lucidum	45	179.2e	632.7de	482.0de	145.6f	150.7a	336.4bcd	108.8 g	233.0a
Lentinula edodes	45	175.2de	673.6f	487.0de	109.1bc	186.5a	377.9de	84.9a	381.5def
Pleurotus citrinopileatus	45	224.7 g	499.8ab	358.5a	94.2a	141.3a	264.3a	131.2i	404.5 g
Pleurotus eryngii	45	165.4bc	612.0 cd	466.2de	112.0bc	145.8a	354.2cde	95.7e	381.9def
Phlebia rufa	45	203.7f	534.6b	401.9b	131.1e	132.7a	270.8a	123.6 h	387.9efg
Autoclaved stover (control)	–	147.5a	643.6def	470.4de	121.5cde	173.2a	348.9cde	94.2de	356.8c
Intact stover	–	143.7a	618.4 cd	474.1de	107.5b	144.3a	366.5cde	91.1 cd	398.8 fg
SEM		1.68	6.87	6.77	2.35	12.32	7.54	0.75	3.50

Values with different letters within a column are significantly ($P < 0.05$) different. DM, dry matter; OM, organic matter; CP, crude protein; NDF, ash-free neutral detergent fiber; ADF, ash-free acid detergent fiber; ADL, acid detergent lignin; HC, hemicellulose; IVDOM, *in vitro* digestibility of organic matter; SEM, standard error of mean.

these inorganic nitrogen sources, degrading them to ammonium ions that can be assimilated into glutamate and glutamine;[29,33] thus the net increase in nitrogen content (Table 2) might be related to the transformation of nitrates and nitrites during fungal incubation, which will then be quantified by the total Kjeldahl N method. Ultimately, the net increase in CP content is considered an advantage of the fungi- treated fibrous substrates.[34] Our data show that for *P. citrinopileatus* the net gain in CP, in comparison with the intact stover, was around 13% at 22 days of incubation (Table 2).

Only *P. citrinopileatus* (22 and 45 days) and *P. rufa* (22 and 45 days) treatments decreased ($P < 0.05$) the NDF and ADF proportion of cowpea stover, while the other treatments did not promote any changes in these cell wall components (Table 1). In relation to the ADL fraction, only the treatment with *P. citrinopileatus* (22 and 45 days) was able to decrease its content ($P < 0.05$), and an increase in the proportion of ADL was even obtained for *G. lucidum* treatment at 45 days of incubation ($P < 0.05$). As reported before,[34] the increase in the contents of this fraction due to fungi treatments can be due to its smaller losses in relation to OM losses (Table 2). Differences obtained in the relative proportions of the different analyzed fractions between 22 and 45 days of incubation might also be explained by an increase in OM losses during the incubation period (Table 2).

All fungi, except *P. eryngii* at 22 days of incubation, caused a net loss of DM and OM (Table 2), with the highest consumption occurring at 45 days of incubation for all fungi treatments ($P < 0.05$). These results were expected, as later development of fungi during the colonization process will implicate the degradation of structural cell wall components compared with the utilization of available soluble compounds during the initial stage. Higher losses ($P < 0.05$) of DM and OM where observed in incubations with *P. citrinopileatus* (22 and 45 days). For cell wall contents NDF, ADF and ADL, higher depletion (between 41 and 46%) was also detected for the treatment with *P. citrinopileatus* ($P < 0.05$). Nevertheless, the degradation pattern between 22 and 45 days of incubation was not similar for all fungi treatments, with no differences identified for *L. edodes*, *P. citrinopileatus* and *P. eryngii* treatments but higher losses ($P < 0.05$) detected for *G. lucidum* and *P. rufa* treatments in NDF and ADF fractions. The incubation period did not influence ADL utilization for any of the fungi treatments ($P > 0.05$). The treatments with *L. edodes* and *P. eryngii* resulted in lower ($P < 0.05$) hemicellulose and cellulose consumption. In contrast, these losses were higher for *P. citrinopileatus* and *P. rufa* treatments ($P < 0.05$). Interestingly, in all fungi, increasing the incubation period did not affect hemicellulose concentrations, and only *G. lucidum* and *P. rufa* treatments increased ($P < 0.05$) cellulose depletion through the incubation period. These changes within the cell wall contents promoted higher ($P < 0.05$) values of *in vitro* digestibility (IVDOM) for all fungi treatments at 45 days of incubation compared with the control, with the exception of *G. lucidum*. In fact, the treatment with this fungus negatively influenced ($P < 0.05$) the IVOMD. At 22 days of incubation, only *P. citrinopileatus* and *P. rufa* showed higher IVOMD values. It should also be noted that only the treatment with *P. citrinopileatus* at 22 days of incubation was able to improve the IVOMD of intact stover by 17%. Furthermore, a decrease in IVOMD was measured for *G. lucidum* and *P. citrinopileatus* along the incubation period.

Various fungal species have been used to valorize different lignocellulosic substrates for animal nutrition. In a recent review, van Kuijk *et al.*[9] highlighted the main biomass sources that can be used as animal feed ingredients after fungal pretreatment, mentioning that these substrates are mainly selected for their potential nutritive value but also because of their geographical availability. These substrates are mainly constituted by grass straws and stovers and residues from other grasses such as bamboo and sugarcane. Data on fungi treatment of legume residues are scarce, and to our knowledge there are no references on legume straws. Furthermore, owing to their nutritional characteristics, these substrates have been evaluated as ruminant feeds, and data on rabbit feeding studies are also infrequent.

In general, data from these studies have indicated that fungi-treated material is adequate for use in animal feeding, enhancing changes in the cell wall chemical composition and digestibility.[31,34–38] Although the effectiveness of fungi treatments depends on several factors such as the fungal strain, the chemical and structural features of the substrate and the

Table 2. Loss of nutrients (%) in cowpea stover incubated with different fungi for 22 and 45 days compared with control

Fungus	Incubation	DM	OM	CP[a]	NDF	ADF	ADL	HC	Cellulose
Ganoderma lucidum	22	3.7b	4.3b	−9.8bc	7.5a	7.3ab	0.3a	8.0ab	9.7a
Lentinula edodes	22	8.4c	7.8c	−1.1de	4.7a	4.3ab	15.4bc	5.8a	0.4a
Pleurotus citrinopileatus	22	19.6f	22.1f	−13.0a	41.0d	40.9f	46.1d	41.5b	39.0c
Pleurotus eryngii	22	0.0a	0.0a	−2.5de	3.0a	0.5a	3.1ab	11.3ab	0.1a
Phlebia rufa	22	8.7c	9.1c	−7.3c	15.9b	17.7d	9.2abc	38.4ab	20.6b
Ganoderma lucidum	45	17.4e	18.7e	−0.3e	18.8b	15.6 cd	1.1ab	27.5ab	20.7b
Lentinula edodes	45	12.6d	13.9d	−3.7d	8.6a	9.9bd	21.7c	5.0a	5.8a
Pleurotus citrinopileatus	45	27.0 g	30.0 g	−11.1ab	43.3d	44.6f	43.5d	39.9ab	45.0c
Pleurotus eryngii	45	2.6b	2.8b	−9.1bc	7.4a	3.9ab	10.4abc	17.3ab	1.6a
Phlebia rufa	45	20.3f	22.9f	−10.0bc	33.8c	32.2e	14.1abc	38.4ab	38.5c
SEM		0.24	0.36	0.50	1.01	1.21	2.61	6.27	1.83

Values with different letters within a column are significantly ($P < 0.05$) different. DM, dry matter; OM, organic matter; CP, crude protein; NDF, ash-free neutral detergent fiber; ADF, ash-free acid detergent fiber; ADL, acid detergent lignin; HC, hemicellulose; SEM, standard error of mean.
[a] The negative sign indicates an *increase* in proportion.

culture conditions, it has been proposed that fungi should be selected according to their specificity in degrading lignin without substantially depleting cellulose and hemicellulose concentrations, as these structural carbohydrates may be extensively used by herbivore animals, increasing the digestibility of the substrate.[9] The data presented in this study clearly show that there are differences within the fungal degradation mechanisms, indicating that *P. citrinopileatus* treatment was the most effective in enhancing IVOMD. Although it may be argued that this is due to the decrease in ADL contents, the high losses of hemicellulose and cellulose during this treatment do not agree with previous data reported by Tuyen *et al.*[34] The results obtained for *P. rufa* treatment also point to higher *in vitro* digestibility values in spite of high losses of hemicellulose and cellulose fractions. Tuyen *et al.*[34] pointed out that for wheat straw, only fungi characterized by high lignin degradation potential but low depletion of cellulose would be able to improve its nutritive value. Other authors working with the same fungi used in this study have also reported different results.[24,30,31,39] Again, differences in the degradation patterns of cell wall components might be attributed to morphological differences within the cell wall structure of the substrates used in the different experiments or to any of the other above-mentioned factors. However, it should be noted that the lignin composition of legumes comprises only guacyl and syringyl units, while that of grasses also possesses high amounts of *p*-hydroxyphenyl units.[10] Furthermore, legumes have smaller amounts of ferulate and *p*-coumarate esters,[40] phenolic acids that allow the formation of linkages between lignin and carbohydrates, thus influencing the fungal degradation patterns. In addition, NDF is also lower in legumes, while lignin is present in higher concentrations.[41] Moreover, no ferulate-mediated crosslinks of lignin to cell wall polysaccharides have been observed in legumes.[42] Therefore the variations in cell wall degradation observed in studies using fungal incubation in legume substrates might not be similar to the results obtained when using grasses.

Some studies have pointed out the advantages of prolonged incubation times that will positively influence the nutritive value of substrates,[31,34,43] showing an increase in the *in vitro* digestibility and/or gas production, in spite of higher DM losses that might compromise the efficacy of utilization of these fungi.[8] In contrast, Shrivastava *et al.*[30] identified higher *in vitro* gas production and IVOMD for shorter incubations times (10 and 20 days) in wheat straw treated with *Pleurotus ostreatus* and *Trametes versicolor* respectively. Also, Lynch *et al.*,[44] evaluating the effect of the same fungal species on the DM and NDF digestibility of maize stovers, only reported a positive effect for *P. ostreatus*. Therefore it seems that the influence of the incubation period might depend on the nature of the substrate, the incubation procedures and the specific fungi growth patterns. Our results also confirm these data, showing that fungi present different colonization strategies during the incubation period. Thus the most effective treatment was attained at 22 days of incubation for *P. citrinopileatus*, allowing for an increased digestibility of around 30.4% compared with the control. In a recent study, van Kuijk *et al.*[45] also describe this problem, showing that results could even vary depending on the utilization of different batches, cultivars and growth conditions of substrates.

CONCLUSIONS

Data presented in this study show clear differences between the colonization patterns of fungi on cowpea stover. The chemical composition of the substrate and the incubation conditions used allowed the *P. citrinopileatus* strain an optimal growth and a more efficient delignification process, which resulted in higher *in vitro* digestibility. This work highlights the need for future work in order to optimize the incubation process, seeking to increase the lignin degradation efficiency while limiting the degradation of cellulose and hemicellulose.

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