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Assessing the nutritional quality of fungal treated wheat straw: Compounds formed after treatment with *Ceriporiopsis subvermispora* and *Lentinula edodes*

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ABSTRACT

A variety of secondary metabolites are formed and compounds released during the bioconversion of lignocellulosic biomass by white-rot fungi that can affect the nutritional value and acceptance of the biomass by ruminants. Changes in pH, ergosterol content, fibre and metabolites composition of wheat straw (WS) incubated with either *Ceriporiopsis subvermispora* or *Lentinula edodes* for up to 8 weeks were investigated. With increases in mycelium content, significant decreases in absolute amount of hemicellulose, acid detergent lignin and, to a lesser extent, cellulose were observed in both fungal treatments. Acidification mainly occurred within the first four weeks of incubation, coinciding with the largest changes in metabolites profile. Diverse compounds, including organic acids and soluble sugars increased or decreased with *C. subvermispora* and *L. edodes* treatment. None of the thirty-four common mycotoxins analyzed were detected in WS after 8 weeks of fungal incubation. These results provide important information for application of fungal treated WS that might affect animal acceptance and performance.

1. Introduction

White-rot fungi are considered the most effective microbiota to decay highly lignified biomass and have been employed to increase the availability of structural polysaccharides (cellulose and hemicellulose) for other microbiota, such as those in the rumen (Van Kuijk et al., 2015). These fungi produce a mixture of peroxidase and auxiliary enzymes that break C—C and ether linkages in lignocellulosic waste, increasing the availability of polysaccharides for several down steam processes (Dilokpimol et al., 2016). The degradation capacity of white-rot fungi depends on the fungal species/strain, substrate, incubation conditions and selected additives (Tuyen et al., 2012, 2013; Van Kuijk et al., 2016a; Nayan et al., 2018). Some fungi are classified as non-selective and degrade lignin as well as polysaccharides. Selective fungi degrade lignin and hemicellulose but little to no cellulose. *Ceriporiopsis subvermispora* and *Lentinula edodes* belong to the latter group and have been shown to be the most effective white-rot fungi to increase the fermentability of wheat straw (WS) by rumen microbes (Tuyen et al., 2012; Mao et al., 2018, 2020).

Stability and safety are important criteria for the use of fungal treated substrates as animal feed. A wide variety of secondary

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metabolites are formed, or compounds are released from lignocellulosic biomass by white-rot fungi during bioconversion. Both can affect the nutritional value and acceptance of the treated biomass by ruminant animals. Previous studies have shown that *C. subvermispora* and *L. edodes* produce a series of organic acids and hence acidify the substrate (Mäkelä et al., 2002; Kwak et al., 2016) as well as release free sugars during colonization of lignocellulosic biomass. In addition, *C. subvermispora* produces different organic acids compared to most other white-rot fungi, *i.e.* ceriporic acids (Enoki et al., 2002; Amirta et al., 2003; Nishimura et al., 2008, [Nishimura et al.,2012a]2012a and b), which are important for cell wall degradation. Different types of organic acids are produced by these fungi in the process of degradation of lignin and an analysis of these and other compounds will improve our understanding of lignin degradation. Moreover, detailed chemical analyses provides further insight into the quality and safety of fungal treated lignocellulosic biomass as an animal feedstuff.

Some filamentous fungi are reported to produce mycotoxins as secondary metabolites, whereas white-rot fungi are known to have detoxification capabilities (Wang et al., 2011; Yehia, 2014). Mycotoxins, when present in feeds, can significantly affect animal performance, health and welfare (Gallo et al., 2015). *L. edodes* is an edible and medicinal fungus that has been widely used in the cultivation of edible mushrooms and is considered safe for consumption. *C. subvermispora*, however, is a crust fungus which does not produce harvestable mushrooms for consumption and has sporadically been used in feeding studies.

This study determined the changes in selected organic compounds during 8 weeks of treatment of WS by *C. subvermispora* and *L. edodes,* using gas chromatography mass spectrometry (GC–MS). Liquid chromatography mass spectrometry (LC–MS) was employed to determine four ceriporic acids (A, B, C and G) while the presence of 34 commonly analysed mycotoxins in *C. subvermispora* and *L. edodes* treated WS was also evaluated.

2. Materials and methods

2.1. Fungal strain and spawn preparation

The white-rot fungi *C. subvermispora* (CBS 347.63; USA) and *L. edodes* (CCBAS389; Czech Republic), maintained in liquid nitrogen (N) at the mushroom breeding laboratory of Wageningen University & Research (Wageningen, The Netherlands), were used. The fungi were grown on malt extract agar plates, containing 10 g L^{-1} malt extract (Oxoid Ltd, Hampshire, UK) and 17.5 g L^{-1} micro agar (Duchefa Biochemie B.V, Haarlem, The Netherlands), at 25 °C for 1–2 weeks (depending on the fungus) until the entire plates were fully colonized with mycelium. Two pieces (~ 1.5 cm^2) of agar were then transferred to plastic boxes (OS60 + OD60; Combiness, Nevele, Belgium) with approximately 75 g of autoclaved sorghum grains and incubated at 25 °C until the fungi fully colonized the grains. The spawn was then maintained at 4 °C for further inoculation on wheat straw (WS).

2.2. Fungal inoculation and incubation

Dry organic WS was mechanically chopped to 0.5–2.0 cm with a chop machine (Pierret Industries, Corbion, Belgium), filled in monofilament net bags (50 × 80 cm, pore size: 1.5 × 1.5 mm) and immersed in tap water for three days to attain enough moisture to meet the growth requirement of fungi. The straw was drained for 5 h and mixed by hand. The fungal treatments were carried out in 1.2 L plastic boxes (TP1200 + TPD1200; Combiness, Nevele, Belgium), with 240 g wet WS in each box, before autoclaving at 121 °C for 1 h. The dry matter (DM) content of the autoclaved WS inoculated with fungal spawn at week 0 were 224.7 (*C. subvermispora*) and 228.9 g kg^{-1} wet substrate (*L. edodes*). All boxes were left overnight in a flow cabinet for further fungal inoculation. The autoclaved WS was inoculated with 6 g of each fungal spawn, and 18 boxes of each fungal species were then maintained at 25 °C in a climate-controlled room for 8 weeks. Three boxes of each fungal treated WS were collected at week 0, 1, 2, 4, 6 and 8 of incubation, and stored in a freezer for further freeze drying and analysis. The weight of each box was recorded to calculate the absolute amount of cell wall components during fungal treatment.

2.3. Cell wall composition and pH analysis

All substrate in the boxes were freeze dried and milled to pass a 1 mm sieve (100 AN; Peppink, Olst, The Netherlands). The DM content was determined at 103 °C for 4 h according to the methods described in ISO 6496 (1999), where after the dried samples were incinerated in a muffle furnace at 550 °C for 3 h to determine the ash content (ISO 5984, 2002). The N content was also determined according to ISO 5983 (2005). Fibre fractions were determined as described by Van Soest et al. (1991), using an automatic Ankom fiber analyzer (A2000I; ANKOM Technology, Macedon, NY, USA). Briefly, neutral detergent fibre (aNDFom) was determined by boiling the dried material in neutral detergent with the addition of a heat-stable amylase (Thermamyl) and alcalase. Acid detergent fibre (ADFom) was determined by boiling the dried material in acid detergent (AD). Acid detergent lignin (solubilize cellulose with sulphuric acid; lignin (sa)) was obtained by incubating AD boiled dry material in 72 % sulphuric acid. In the end, the residue was washed, dried, weighed and incinerated. The loss in mass resulting from incineration of the dried residue corresponds to the mass of fibre. The cellulose content was calculated as the difference between ADFom and Lignin (sa), and hemicellulose as the difference between aNDFom and ADFom. The absolute amount of the cell wall components was calculated based on the weight change of the fungal treatment at the beginning and the end of the incubation. The pH of the dried and ground samples was measured in a titrando machine (Metrohm, Herisau, Swizerland) after adding 30 mL demineralised water to 1 g of sample and mixing in a shaker.

2.4. Ergosterol measurement

The ergosterol content of the freeze-dried samples was determined as previously described by Niemenmaa et al. (2008). Briefly, approximately 0.2 g sample was weighed into a glass tube to which 3 mL of 10 % KOH in methanol solution was added. The suspension was mixed and saponified at 80 °C for 60 min. As an internal standard, 20 μL of cholecalciferol (vitamin D_3, 0.5 μg μL^{-1}) was added, followed by addition of 1 mL of H_2O and 2 mL of hexane after the suspension was cooled down to room temperature. The hexane phase was transferred to a new glass tube after centrifuging (4000 rpm for 10 min) of the suspension where after the extraction procedure with water and hexane was repeated. The collected hexane phases were pooled and the hexane was evaporated under vacuum (30 °C for 60 min). The residue was dissolved in 1 mL of methanol and vortexed for 5 s. The solution was filtered and transferred to a glass vial with insert for HPLC analysis (Alliance HPLC system, Milford, USA) which consisted of a Waters HPLC-PDA system and a Phenomex aqua 5 μm C18 column (250 × 4.6 mm). The solvent was 90 % methanol and 10 % (1:1) 2-propanol/hexane, with a flow rate of 0.5 mL/min and measurement of ergosterol at 280 nm.

2.5. Metabolite profiling in fungal treated wheat straw

The relative contents of polar metabolites in fungal treated WS samples were determined by GC–MS according to the method described by Lisec et al. (2006). Modifications were performed enabling a better extraction of compounds from the specific freeze-dried material. Briefly, approximately 50 mg (48−52 mg) ground sample was extracted in a 2 mL Eppendorf tube with 1.4 mL of 80 % pre-cooled methanol (−20 °C) and mixed with 60 μL of ribitol (0.2 mg mL^{-1}) as internal standard. After shaking in a thermomixer at 950 rpm for 10 min at 70 °C (Vortemp 56; Labnet International Inc., Edison, USA) and centrifuging in a Eppendorf centrifuge for 10 min at maximum speed (21,000 × g), 500 μL of the supernatant was transferred into an new Eppendorf tube and mixed with 375 μL of chloroform and 750 μL of H_2O for 10 s. The phase was separated by centrifuging at max speed (21,000 × g) in an Eppendorf centrifuge and 100 μL of the methanol-water phase was transferred into an 180 μL glass insert in a 2 mL vial. All the extracts were dried by vacuum centrifugation.

The residues were derivatized online using a Combi PAL autosampler (CTC Analytics AG; http://www.ctc.ch). The compounds were analysed *via* the method described and adapted by Carreno-Quintero et al. (2012). Two μL of the derivatized samples were analysed by a GC–MS system consisting of an Optic 3 injector (ATAS GL Int., Eindhoven, the Netherlands) and an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments, Inc., St. Joseph, MI, USA).

Ceriporic acids A, B, C and G were analyzed by LC coupled to an LTQOrbitrap FTMS hybrid system (LC–MS). The compounds were extracted using a protocol described by De Vos et al. (2007). Briefly, ~ 100 mg (97−103 mg) of sample powder was weighed and extracted in a 2 mL Eppendorf tube with 1.5 mL of 75 % methanol containing 0.1 % formic acid. The extract was vortexed (10 s) and then sonicated for 10 min. The phase was separated by centrifuging for 10 min in an Eppendorf centrifuge (16,000 × g), and 180 μL of the supernatant was transferred to an insert glass vial for LC–MS analysis. Chromatographic separation, mass detection methods and LC–MS equipment were as described by Mokochinski et al. (2018). Ceriporic acid A, B and C were identified by matching their observed specific accurate masses and mass spectra with those provided by Amirta et al. (2003) and Van Kuijk et al. (2017), while the mass spectrum of ceriporic acid G was according to Nishimura et al. (2012b).

2.6. Mycotoxin analysis

The mycotoxin content was analysed using a validated and accredited LC–MS/MS-based analytical method at the Dutch National Reference Laboratory RIKILT (Wageningen University & Research, Wageningen, The Netherlands). Prior to the analysis, 0.9 g of the substrate was weighed in a 50 mL plastic tube and 6 mL of water, 10 mL of extraction solvent (acetonitrile/acetic acid 990:10 (v/v)), and 25 μL of ^{13}C-caffeine internal standard were added. The suspension was shaken manually and placed in an extracting device (Heidolph Reax 2, Schwabach, Germany) for 30 min. Four g of magnesium sulphate were added, the tube vortexed for one min and centrifuged at 3000 rpm for 10 min. Two hundred μL of extract was transferred to polypropylene vials (Whatmann syringeless filter device) and diluted with 200 μL water, capped and shaken in a vortex mixer for approximately 3 s. After 30−60 min in the refrigerator (4 °C), vials were closed with a pressing device (Six Position Compression; Whatman, 's-Hertogenbosch, The Netherlands) and stored at 4 °C until analysed by a LC–MS/MS (Waters Acquity, Etten-Leur, the Netherlands; AB SCIEX QTRAP® 6500, Applied Biosystems, Nieuwekerk aan de IJssel, the Netherlands) with a Restek Ultra Aqueous C18 3 μm 100 × 2.1 mm UPLC column (Restek Corporation, Bellefonte, USA) and a sample injection volume of 5 μL. The gradient was achieved using two different eluents: Eluent A consisted of 1 mM ammonium formate and 1% (v/v) formic acid in water; Eluent B consisted of 1 mM ammonium formate and 1% (v/v) formic acid in a mix of methanol and water 95:5 (v/v). Eluent C consisted of 5 mM ammonium acetate and 0.1 % (v/v) acetic acid in water; Eluent D consisted of 5 mM ammonium acetate and 0.1 % (v/v) acetic acid in a mix of methanol and water 95:5 (v/v). Eluent A and B were used in the positive (ESI+) and C and D in the negative (ESI-) mode. Each mycotoxin was identified by its retention time and the peak area ratio between two transitions (Table S1). For nitropropionic acid and moniliformin, however, one transition was available. Therefore, identification of these mycotoxins was regarded to be tentative. Quantification was performed by a single point standard addition protocol. Validation parameters can be found in the supplementary Table (Table S1).

2.7. Data processing and statistics analysis

The GC–MS data were processed in an untargeted manner according to the steps performed by Mokochinski et al. (2018). In short, Metalign software was used for unbiased mass peak picking and alignment, after which MSClust software was used to assemble all mass signals originating from the same metabolite. The relative intensity of the resulting clusters (putative metabolite) were used in subsequent data analyses. Principal component analysis (PCA) was performed using the software Simca (version 14) after log-transformation and Pareto-scaling of the relative abundance values of the metabolites. Mass spectra of clusters, together with their observed retention index, were used to annotate selected compounds by comparing data obtained with authentic standards and the dedicated Golm EI-spectral database (http://gmd.mpimp-golm.mpg.de).

The pH, cell wall composition, ergosterol and ceriporic acid were analysed in SAS 9.4 using the generalized linear model (GLM):

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where Y_{ij} is observation j in treatment i, μ is the overall mean, α_i is the fixed effect of time and ε_{ij} is the random error. Multiple comparisons using Tukey's significant test with $\alpha = 0.05$ was used to determine significance between treatments. The pH and cell wall composition data contained biological triplicates while ergosterol data consisted of two of the biological triplicates. For GC–MS data, independent sample t-tests in SAS, version 9.4, were used to compare the difference between week 0 and 8 after fungal incubation.

3. Results

3.1. pH and mycelium growth of fungal strains

The extracts of WS during 8 weeks of treatment with *C. subvermispora* and *L. edodes* showed a decrease in pH (Fig. 1A). *C. subvermispora* reduced ($P < 0.05$) the pH of the autoclaved WS from 4.75 at the start to 3.30 after 8 weeks whereas *L. edodes* reduced ($P < 0.05$) the pH from 4.74 to 3.92. *C. subvermispora* showed a linear growth ($P < 0.05$) in the first 6 weeks (up to 92.2 μg ergosterol g^{-1} dry substrate) of incubation after which the fungal biomass remained relatively constant (91.8 μg ergosterol g^{-1} dry substrate) ($P > 0.05$). *L. edodes* showed a lag phase of growth in the first week after which growth increased ($P < 0.05$) from 4 (92.5 μg ergosterol g^{-1} dry substrate) to 8 weeks of incubation (270.9 μg ergosterol g^{-1} dry substrates) (Fig. 1B).

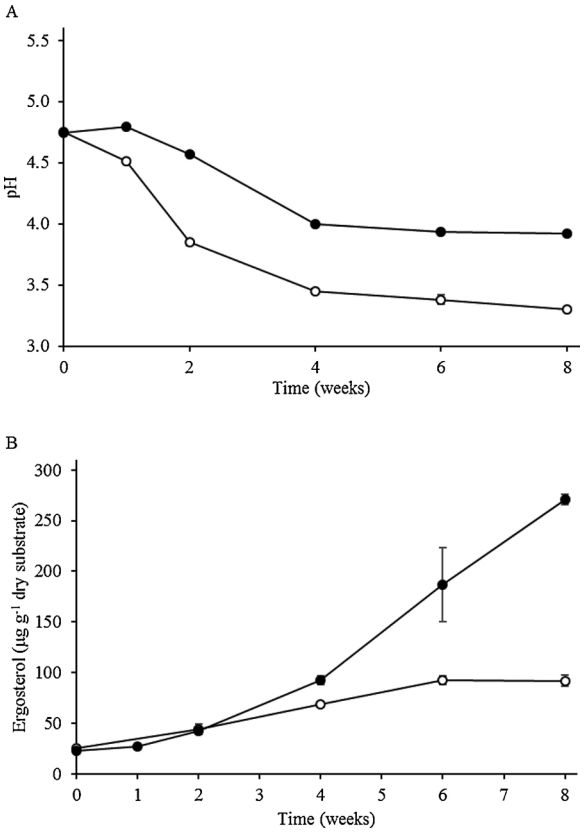

Fig. 1. pH (A) and ergosterol content (B) of autoclaved wheat straw treated with *Ceriporiopsis subvermispora* (○) and *Lentinula edodes* (●) for 0, 1, 2, 4, 6 and 8 weeks. Error bars indicate standard deviations.

Table 1
Cell wall components of wheat straw cultured with *Ceriporiopsis subvermispora* for 0 to 8 weeks.

Duration (Week)	Concentration (g kg^{-1} DM)					Absolute amount (g per 240 g starting wet substrate)					
	Ash	N	Cellulose	Hemicellulose	Lignin (sa)	DM	Ash	N	Cellulose	Hemicellulose	Lignin (sa)
0	40.1^{c}	3.27^{c}	470.9^{c}	280.2^{a}	73.0^{b}	52.9^{a}	2.12	0.173	24.9^{a}	14.8^{a}	3.86^{b}
1	41.9^{bc}	3.31^{c}	466.1^{c}	280.5^{a}	81.9^{a}	52.4^{a}	2.20	0.173	24.4^{ab}	14.7^{a}	4.30^{a}
2	41.3^{bc}	3.50^{bc}	475.5^{c}	237.4^{ab}	68.0^{b}	50.9^{b}	2.11	0.178	24.2^{bc}	12.1^{b}	3.46^{c}
4	44.2^{ab}	3.57^{bc}	492.3^{b}	192.5^{b}	40.9^{c}	48.3^{c}	2.13	0.172	23.8^{cd}	9.28^{c}	1.97^{d}
6	45.8^{a}	3.80^{ab}	516.0^{a}	133.5^{c}	29.9^{d}	45.3^{d}	2.08	0.172	23.4^{d}	6.05^{d}	1.36^{e}
8	47.6^{a}	4.06^{a}	522.3^{a}	115.7^{c}	24.3^{e}	44.5^{e}	2.12	0.181	23.2^{d}	5.15^{d}	1.08^{e}
RMSE	1.33	0.13	3.58	19.9	1.97	0.27	0.07	0.01	0.23	0.95	0.10
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.535	0.389	<0.001	<0.001	<0.001

DM, dry matter; N, nitrogen; Lignin (sa), acid detergent lignin; RMSE, root mean square error.
[a−e] Means with different superscripts within a column are significantly different ($P < 0.05$), n=3.

3.2. Cell wall composition

After 8 weeks of both fungal treatment, the concentration (g kg^{-1} DM) of ash, N and cellulose were increased ($P < 0.05$) while the concentration of hemicellulose and lignin (sa) were decreased ($P < 0.05$) (Tables 1 and 2). The absolute amount (g per 240 g starting wet substrate) of ash and N content of WS did not change ($P > 0.05$) during the 8 weeks of treatment by *C. subvermispora* or *L. edodes*. A decrease ($P < 0.05$) in the absolute amount of DM was observed for both fungal treatments, with 15.9 % and 17.4 % DM loss after 8 weeks treatment with *C. subvermispora* and *L. edodes*, respectively. The decrease in DM was mainly caused by a decrease in hemicellulose and lignin. There is a clear difference between the fungi in selectivity of degradation.

3.3. GC–MS and LC–MS analyses of fungal treated wheat straw

Metabolite profiling was performed on the different fungal treated WS samples. To provide a general impression of changes in the overall composition of the polar metabolite profile in time and between the two fungi, PCA was performed on the GC–MS data. The first two principal components, indicated by (R2x[1] and R2x[2]), described 52.1 % of the total GC–MS metabolite variation (Fig. 2). The analysis of samples at weeks 0, 4 and 8 were conducted in biological triplicates, the other time point measurements were on single biological samples. The relative low variation between the replicates indicates a relative high reproducibility of the experimental and analytical methods used. A clear distinction is seen between the incubation time points and fungal strains (Fig. 2). The largest change in metabolite profile from the GC–MS analysis mainly occurred between week 0–4 for both fungi (Fig. 2).

The compounds that were identified were classified into three groups: organic acids, carbohydrates/polyols and others (Table 3). Since no analysis was performed on dry WS before soaking in water, we cannot exclude that some organic acids were already present in WS. High levels of lactic and succinic acid observed in WS inoculated with the two fungal strains at week 0 was reduced ($P < 0.05$) after 8 weeks with very negligible residual lactic and succinic acid. As seen with other basidiomycetes, oxalic acid accumulated ($P < 0.05$) during the *C. subvermispora* and *L. edodes* treatment, and was produced in similar amounts by both fungi after 8 weeks of treatment. A higher accumulation of vanillic acid was seen in *C. subvermispora* than in *L. edodes*, while levels of protocatechuic acid decreased ($P < 0.05$) after 8 weeks in both fungal treatments. Both fungi differed in the amount of organic acid produced. *C. subvermispora* produced more formic and tartaric acid while *L. edodes* produced more malic and galacturonic acid. A clear difference in organic acids between the fungi were also the ceriporic acids, which were only produced by *C. subvermispora*. Four ceriporic acids (A, B, C and G) were identified by LC—MS (Fig. 3). These compounds accumulated ($P < 0.05$) until four weeks of *C. subvermispora* incubation and remained stable or slightly decreased thereafter.

Especially striking are the relatively high levels of mono- and disaccharides at the start of the incubation (Table 3). For *C. subvermispora*, the xylose and fructose levels decreased sharply within the first week of incubation (Table 3, Table S2). In the case of *L. edodes*, those compounds degraded sharply after 2 weeks. Glucose level, however, decreased only after 2 weeks of incubation in both fungal treatments and was especially high in the first 2 weeks of *L. edodes* treated WS. Remarkable is the accumulation of the major polyols by both fungi. The relative level of arabitol and mannitol in *L. edodes* treated WS at week 8 was approximately 3 and 10 times higher, respectively than in *C. subvermispora*. Glycerol, on the other hand, increased ($P < 0.05$) only in *C. subvermispora* treated straw and *L. edodes* treated WS showed a strong decrease ($P < 0.05$).

3.4. Mycotoxin content

Although it is unlikely that the basidiomycetes used here produce known mycotoxins, samples of untreated WS and 8 weeks fungal treated WS were analysed for the presence of known mycotoxins (Table 4). Except for Enniatin B in untreated WS, none of the other mycotoxins could be detected in untreated and treated WS.

Table 2
Cell wall components of wheat straw cultured with *Lentinula edodes* for 0 to 8 weeks.

Duration (Week)	Concentration (g kg^{-1} DM)					Absolute amount (g per 240 g starting wet substrate)					
	Ash	N	Cellulose	Hemicellulose	Lignin (sa)	DM	Ash	N	Cellulose	Hemicellulose	Lignin (sa)
0	39.4^{d}	3.27^{b}	461.2^{e}	273.6^{a}	70.4^{a}	53.4^{a}	2.11	0.175	24.6^{ab}	14.6^{a}	3.77^{a}
1	40.0^{d}	3.31^{b}	474.4^{d}	259.1^{b}	66.0^{b}	53.0^{ab}	2.12	0.176	25.2^{a}	13.8^{b}	3.50^{b}
2	40.6^{cd}	3.43^{b}	477.1^{d}	258.6^{b}	70.7^{a}	52.4^{b}	2.13	0.180	25.0^{a}	13.6^{b}	3.71^{ab}
4	42.4^{c}	3.46^{b}	497.7^{c}	199.0^{c}	58.9^{c}	49.5^{c}	2.10	0.172	24.7^{a}	9.87^{c}	2.92^{c}
6	44.8^{b}	3.60^{ab}	512.9^{b}	175.9^{d}	51.6^{d}	46.8^{d}	2.10	0.169	24.0^{bc}	8.24^{d}	2.42^{d}
8	46.8^{a}	3.83^{a}	532.9^{a}	156.2^{e}	45.7^{e}	44.1^{e}	2.07	0.169	23.5^{c}	6.89^{e}	2.01^{e}
RMSE	0.70	0.13	4.07	4.46	1.50	0.26	0.03	0.01	0.23	0.23	0.09
p-value	<0.001	0.0020	<0.001	<0.001	<0.001	<0.001	0.342	0.381	<0.001	<0.001	<0.001

DM, dry matter; N, nitrogen; Lignin (sa), acid detergent lignin; RMSE, root mean square error.
$^{a-e}$ Means with different superscripts within a column are significantly different ($P < 0.05$), n=3.

4. Discussion

A number of studies reported acidification and organic acid production by white-rot fungi in a liquid or solid culture environment (Mäkelä et al., 2002; Kwak et al., 2016). Since the analysis was done on freeze-dried samples, the pH decrease is caused by non-volatile organic acids. In a previous study (Mao et al., 2018), pH was measured in fresh samples and a similar pH profile was observed during fungal incubation. The only difference was that in fresh samples, an initial increase in pH was observed in the first week with *C. subvermispora*, which might have been caused by volatile acids. This increase was also observed by Zadražil (1977) with other white-rot fungi. Ergosterol is a sterol almost exclusively found in fungal cell membranes, therefore, ergosterol is a good indicator of fungal biomass production (Niemenmaa et al., 2008). The increase of ergosterol content over time indicates that both *C. subvermispora* and *L. edodes* colonized the WS well, but *L. edodes* formed more biomass as could also be seen visually on the inoculated WS. *Ceriporiopsis subvermispora* produced either more or stronger organic acids, especially in the first two weeks, where both fungi had a similar mycelium content, a greater decrease in pH was seen than with *L. edodes*. Whereas for *C. subvermispora*, the pH decrease coincides with the formation of biomass, the growth of *L. edodes* continuous after a pH plateau is reached. As in previous experiments (Nayan et al., 2018; Mao et al., 2018), the strategy of *C. subvermispora* seems to be a quick colonization of the substrate, and once present in most parts, continuous to produce acids to degrade lignocellulose, *i.e.* degrade lignocellulose, without forming more biomass. A balance between production and turnover can explain a constant pH after ceasing of fungal growth. *L. edodes* seems to need growth in order to produce acids. Here also a balance between production and turnover can explain the plateauing of the pH. Since *C. subvermispora* produces more/stronger acids than *L. edodes*, the final pH for the former is lower than the latter. A larger reduction in pH with a lower amount of fungal biomass indicates that *C. subvermispora* is more efficient in affecting organic biomass than *L. edodes* and is able to degrade lignin to a higher extent, as is shown in Tables 1 and 2. This is underlined by observations in a study by Blanchette et al. (1997) where they showed that even with the presence of single hypha of *C. subvermispora* in a plant cell wall lumen, a clear degradation/alteration of the cell wall is seen.

The absolute amount of N in WS did not change across the incubation period indicating that N remains in WS and is incorporated into fungal biomass. During growth, the fungi convert the carbon source partly into new fungal biomass and partly into CO_2 (which escapes the substrates). One might thus expect an increase in total nitrogen due to the reduction of organic matter. The total fungal biomass is, however, very small compared to the total substrate mass (2%) and a significant increase in total N is not seen. The low mineralization of cellulose was also observed in previous experiments (Tuyen et al., 2012; Mao et al., 2018; Nayan et al., 2018) and is common for selective white-rot fungi (Martínez et al., 2011). The structural carbohydrates and lignin (sa) were determined using the detergent fibre analysis methods by Van Soest et al. (1991). The method is relatively rapid and useful to identify trends in changes in cell wall fibres. The method is, however, designed for feed analysis and especially the measurement of carbohydrates can be inaccurate since these are measured as residues after extraction, thus as insoluble components. As a consequence, the reduction in hemicellulose measured here is likely, for a part, due to the solubilisation and not due to fungal metabolism (Nayan et al., 2019). That is also underlined by the fact that the decrease in absolute amount of the sum of fractions measured (nitrogen, cellulose, hemicellulose and lignin) is larger than the absolute decrease in organic matter (Tables 1 and 2). The content of cellulose can also be overestimated due to glucans in fungal cell walls (Nayan et al., 2019), while lignin (sa) does not represent the total lignin content, due to the fact that acid soluble lignin is lost with this method (Godin et al., 2014). The detergent fibre method for measuring lignin (sa) is, however, a good estimate for the more "recalcitrant" insoluble lignin that correlates well with *in vitro* degradation in rumen fluid (Nayan et al., 2019). *Ceriporiopsis subvermispora* showed a more rapid reduction in lignin (sa) and to a higher extent than *L. edodes*. Previous research has shown that this also leads to a higher *in vitro* digestibility (Tuyen et al., 2012; Mao et al., 2018; Nayan et al., 2018). The cellulose content (measured as ADFom-Lignin (sa)) is slightly reduced by both fungi whereas the hemicellulose content (measured as aNDFom-ADFom) is strongly reduced and, as mentioned previously, at least in part, due to solubilisation. An increase in solubilisation

of hemicellulose might also be due to a higher degradation of lignin (sa) which is covalently bound to hemicellulose (Hatakka and Hammel, 2011; Nishimura et al., 2018). The loss of OM during the 8 weeks of incubation is very similar for both fungi. One would expect that the OM loss for *L. edodes* would be higher since more fungal biomass is formed, whereby approximately half of the carbon is converted to new fungal biomass and the other half to CO_2. The amount of fungal biomass formed, however, represents only a small part of the total DM (Nayan et al., 2019) and might be below the detection level.

The difference in metabolite profile between *C. subvermispora* and *L. edodes* treatment at week 0 originates likely from the added spawn at the beginning of the incubation. *Lentinula edodes* degrades the starch of spawn to a much greater extent than *C. subvermispora* (Van Kuijk et al., 2016b). The trend in the change of metabolic profiles of the two fungi in time correlates well with the changes in pH, *i. e.* a more rapid change in *C. subvermispora* treated WS than in *L. edodes* treated WS. Although the measurements for week 1, 2 and 6 are singular, they fit well in this trend.

The lactic acid and succinic acid present at week 0 are likely fermentation products by microorganisms since the three days submersion of WS generates low oxygen conditions. Substrates for fermentation under these conditions can be free sugars that are found in WS (Shan et al., 2008; Tishler et al., 2015). The quick disappearance of these acids indicates metabolisation by the inoculated fungi. Oxalic acid is a common organic acid produced by white-rot fungi in either solid or liquid cultures (Mäkelä et al., 2002; Kwak et al., 2016). Oxalic acid detected in week 0 originates likely from the colonization of fungi on sorghum grains or partly from straw. Vanillic acid and protocatechuic acids are both breakdown products of lignin and different catabolic funnelling pathways involved, have been described (Abdelaziz et al., 2016). The relative higher level of vanillic acid in the WS fermented by *C. subvermispora* corresponds to the better degradation of lignin by this fungus compared to *L. edodes*. Protocatechuic acid is a compound previously reported to be present in WS (Weesepoel et al., 2016) and clearly degraded by both fungi. Galacturonic acid is the main component of pectin. The clear accumulation in samples treated with *L. edodes* and the lack in *C. subvermispora* treated samples indicates a different pathway to degrade pectin. Previous research has shown that when *L. edodes* was grown on microcrystalline cellulose/lignosulfonate, polygalacturonase was one of the most abundant pectinase enzymes produced (Cai et al., 2017). When *C. subvermispora* was grown on Jerusalem artichoke stalks, no polygalacturonase was detected (Zhu et al., 2016). Both fungi produced low molecular weight organic acids that were also previously reported for other white-rot fungi (Liaud et al., 2014) and also found in nature in upper soil levels as degradation products by fungi (Strobel, 2001). These acids play an important role in acidifying the environment and creating an advantage for fungi over bacteria. Organic acids, especially carboxylic acid, also play an important role as a chelator in the degradation of lignin (Kishi et al., 1994). The better degradation of lignin by *C. subvermispora* might be due to its production of ceriporic acids. It is proposed that the lignin degradation is closely related to extracellular lipid peroxidation by *C. subvermispora*, and indeed ceriporic acid G can trigger the lipid peroxidation process catalyzed by manganese peroxide (Nishimura et al., 2012b).

Xylose and arabinose might originate partly from the non-starch polysaccharides in sorghum grains (Knudsen, 2014; Betts et al., 2015) and from WS after autoclaving, as WS contains a considerable amount of free sugars (Shan et al., 2008; Tishler et al., 2015). Fructose, arabinose, xylose, mannose and xylobiose might be produced during the three days of submerging the WS by activity of bacteria and fungi present on the WS. The high amount of glucose at week 0 for *L. edodes* compared to *C. subvermispora* can be explained by the better degradation of starch, present in the added spawn (Betts et al., 2015). *Lentinula edodes* degrades starch much better than *C. subvermispora* (Van Kuijk et al., 2016b). Polyols are widely distributed in fungi (Rast and Pfyffer, 1989). Higher levels of

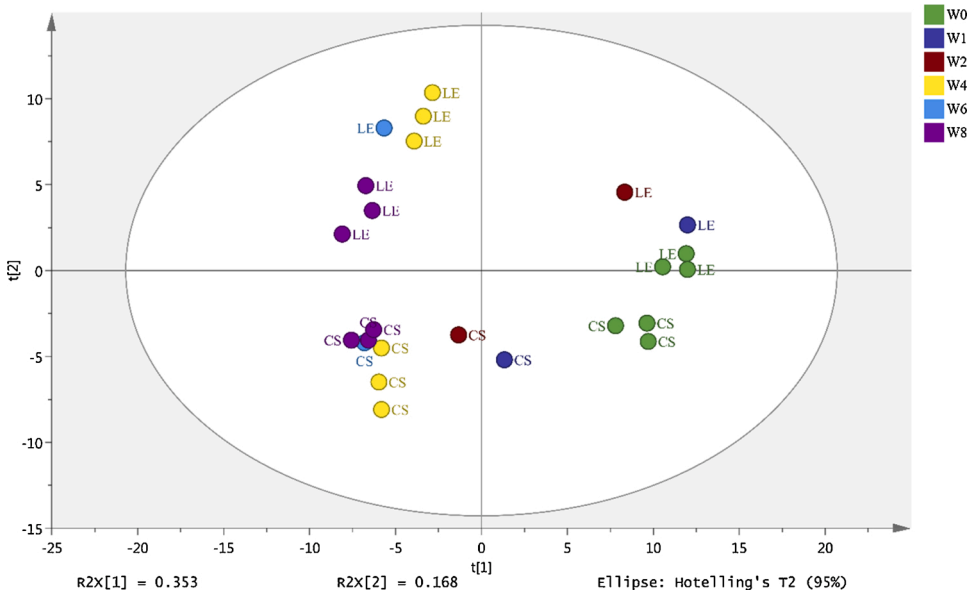

Fig. 2. Principal components analysis showing the major differences in polar metabolites of wheat straw treated with *Ceriporiopsis subvermispora* (CS) or *Lentinula edodes* (LE) for 0, 1, 2, 4, 6 and 8 weeks (W). Polar metabolites were analysed *via* gas chromatography mass spectrometry. Week 0, 4 and 8 analyses were performed in biological triplicates, week 1, 2 and 6 analyses were performed on single biological samples.

Table 3
Compounds (mass-specific ion counts) present in wheat straw treated with *Ceriporiopsis subvermispora* and *Lentinula edodes* for 0 and 8 weeks.

Type	Centrotype	RIexp	Name	Annotation level[†]	*C. subvermispora*		*L. edodes*	
					Week 0	Week 8	Week 0	Week 8
Organic acids	303	1074	Lactic acid, 2TMS	1	23299	201*	26384	76*
	514	1088	Glycolic acid, 2TMS	1	4567	1472*	4785	1349*
	1076	1141	Oxalic acid, 2TMS	1	8050	12496*	6159	10628*
	2828	1301	Succinic acid, 2TMS	1	65043	1131*	61938	1284*
	2965	1312	Glyceric acid, 3TMS	2	3176	3039	3201	2627
	3675	1384	Formic acid, 1TMS	2	383	2766*	369	146
	3756	1392	Malonic acid, 2TMS	2	244	110*	346	66
	4535	1473	Malic acid, 3TMS	1	632	736	615	1574*
	5045	1515	Pipecolic acid, 2TMS	2	757	564*	601	802*
	5362	1543	2,3,4-Trihydroxybutyric acid, 4TMS	2	2109	1628	1870	251*
	6103	1623	Tartaric acid, 4TMS	1	0	1794*	4	39
	8528	1749	Ribonic acid, 4TMS	1	2576	1749*	2542	655*
	8758	1765	Vanillic acid, 2TMS	1	1274	4952*	891	2871*
	9263	1816	Protocatechuic acid, 3TMS	1	7588	375*	7522	133*
	11840	1924	Galacturonic acid, methyloxime 1, 5TMS	1	11	83	0	1351*
Carbohydrates	6170	1627	Arabinose, 4TMS, methyloxime	1	574	2883*	577	1748*
	6502	1636	Arabinose, 4TMS, methyloxime, metyloxime (isomer 2)	1	12254	2380*	12323	475*
	7129	1647	Xylose, 4TMS, methyloxime (anti)	1	95827	1366*	117970	3293*
	7529	1662	Xylose, 4TMS, methyloxime(syn)	1	844	403*	902	229*
	7959	1704	Arabitol, 5TMS	1	26504	70263*	46993	204534*
	9832	1857	Fructose, 5TMS, methyloxime (anti)	1	69554	721*	65696	1065*
	10004	1867	Fructose, 5TMS, methyloxime (syn)	1	56943	310*	52301	479*
	10263	1877	Mannose, 5TMS, methyloxime (1Z)	1	1433	601*	4354	786*
	10566	1883	d-Glucose, 5TMS, o-methyloxyme, (1E)	1	9058	996*	304169	6486*
	11099	1903	d-Glucose, 5TMS, o-methyloxyme, (1Z)	1	2160	177*	70759	1181*
	11450	1914	Mannitol, 6TMS	1	13039	46814*	6202	448761*
	12090	1941	Myo-Inositol, 6TMS	1	9566	5935*	9766	6812*
	13205	2074	Myo-Inositol, 6TMS	2	5758	4733	5650	4221*
	14498	2356	Xylobiose, 6TMS, methyloxime 1	1	396	4994*	454	2324*
	14812	2376	Xylobiose, 6TMS, methyloxime 2	1	104	2034*	125	1292*
	16191	2545	Sucrose, 8TMS	1	20170	13137*	28320	27238
	16763	2625	Maltose, 8TMS, methyloxime (isomer 1)	1	63	0	3512	492*
	16978	2631	Trehalose, 8TMS	1	355	46704*	341	40563*
Others	2308	1257	Glycerol, 3TMS	1	10851	21014*	20369	5650*

TMS, Trimethylsilyl; RIexp, experimentally determined retention index.
[†] According to the rules described by Sumner et al. (2007).
* Significantly different ($P < 0.05$) from week 8 to the corresponding value at week 0.

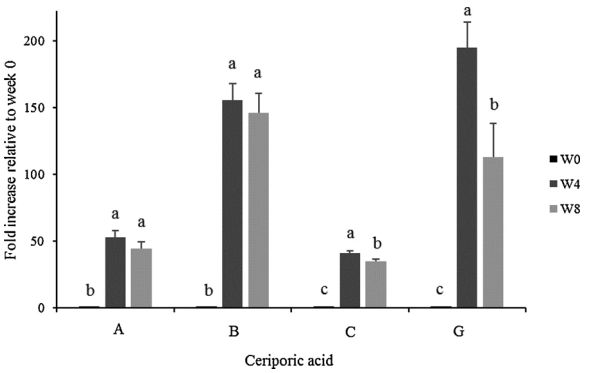

Fig. 3. Fold increase (relative to week 0) in ceriporic acid A, B, C and G produced by *Ceriporiopsis subvermispora* during 8 weeks of incubation on wheat straw. Week 0, 4 and 8 analyses were performed in biological triplicates. Error bars indicate standard deviations. Means with different letters within a ceriporic acid are significantly different ($P < 0.05$).

arabitol and mannitol in *L. edodes* treated WS than in *C. subvermispora* might be explained in part by the difference in the amount of biomass formed. Polyols can be generated by fungi as a carbon storage, but also due to a low water activity or increased osmotic potential of the substrate (Tekolo et al., 2010; Kobayashi et al., 2015), conditions that might have been generated during the 8 weeks of incubation.

The disappearance of the mycotoxin Enniatin B after 8 weeks might indicate that mycotoxins can also be degraded by *C. subvermispora* as has been reported for other white-rot fungi (Wang et al., 2011; Yehia, 2014). The mycotoxin analysis shows that mycotoxins are not an issue in using *C. subvermispora* and *L. edodes* treated WS as an animal feedstuff.

The analysis here shows the potentials of fungal treatment of agricultural waste to increase its digestibility for ruminants. For an application in especially developing countries, where low tech/low costs methods are preferred, further optimizations are needed. Recent experiments indicate that immersion for three days in water is not needed and simpler and shorter methods can be used. Sterilization is also not a practical solution to exclude infection with other fungi. Previous experiments have shown that addition of nitrogen-rich wastes (like different types of bran) and some starch-like material to fibrous agricultural waste stimulate anaerobic fermentation, heating up the substrate which eliminates most unwanted fungi and bacteria (Sonnenberg et al., 2015). This is a good example of a low tech and low cost method.

5. Conclusion

GC–MS analysis showed changes in several organic acids and soluble sugars during *C. subvermispora* and *L. edodes* treatment of wheat straw and there were clear differences between the two fungi. The largest changes in metabolite profiles by both fungi occurred within 4 weeks of incubation, where pH also showed the largest shift. None of the 34 analysed mycotoxins were detected with *C. subvermispora* and *L. edodes* treatment of wheat straw after 8 weeks, indicating that the two fungal treated wheat straws are safe with respect to mycotoxicosis. Those findings are important in the feeding of fungal treated biomass in practice as the analysed components might have either positive or negative effects on animal health, rumen microbes and food safety of treated straw.

Author statement

The concept of the research was done by Lei Mao, Wouter Hendriks, Anton Sonnenberg and John Cone.

The work was carried out by Lei Mao, who was supervised by Wouter Hendriks, John Cone and Anton Sonnenberg.

The biochemical analysis were performed at the laboratory of Bioscience of WUR in Wageningen. That research was also performed by Lei Mao and guided by Jeroen van Arkel and Ric de Vos.

The manuscript was written by Lei Mao and checked by all co-authors.

Table 4
Various mycotoxins (mg kg^{-1} dry sample) analysed[†] in organically grown wheat straw before and after *Ceriporiopsis subvermispora* and *Lentinula edodes* treatment.

Mycotoxin type	Wheat straw	*C. subvermispora*		*L. edodes*	
		Week 0	Week 8	Week 0	Week 8
15-Acetyl-DON	<0.2	<0.2	<0.2	<0.2	<0.2
3-Acetyl-DON	<0.08	<0.08	<0.08	<0.08	<0.08
Aflatoxin B1	<0.004	<0.004	<0.0025	<0.004	<0.0025
Aflatoxin B2, Aflatoxin G1, Agroclavine, Roquefortine C	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025
Aflatoxin G2, Alternariol-methylether	<0.005	<0.005	<0.005	<0.005	<0.005
Alternariol	<0.02	<0.02	<0.02	<0.02	<0.02
Beauvericin	<0.025	<0.025	<0.025	<0.025	<0.025
Citrinin, Enniatin A, Mycophenolic acid, Nitropropionic acid	<0.05	<0.05	<0.05	<0.05	<0.05
Deoxynivalenol	<0.2	<0.2	<0.2	<0.2	<0.2
DON-3-Glucoside	<0.25	<0.25	<0.25	<0.25	<0.25
Diacetoxyscirpenol	<0.0125	<0.0125	<0.0125	<0.0125	<0.0125
Enniatin A1	<0.05	<0.05	<0.05	<0.05	<0.05
Enniatin B	<0.05	0.089*	<0.05	<0.05	<0.05
Enniatin B1	<0.05	<0.05	<0.05	<0.05	<0.05
Fumonisin B1	<0.1	<0.1	<0.1	<0.1	<0.1
Fumonisin B2	<0.04	<0.04	<0.04	<0.04	<0.04
Fumonisin B3, HT-2 toxin, Penicillic acid, T-2 toxin	<0.02	<0.02	<0.02	<0.02	<0.02
Moniliformin	<0.125	<0.125	<0.125	<0.125	<0.125
Nivalenol	<0.1	<0.1	<0.1	<0.1	<0.1
Ochratoxin A	<0.002	<0.002	<0.002	<0.002	<0.002
Sterigmatocystin	<0.001	<0.001	<0.001	<0.001	<0.001
Zearalenon	<0.05	<0.05	<0.05	<0.05	<0.05
α-Zearalenol, ß-Zearalenol	<0.01	<0.01	<0.01	<0.01	<0.01

[†] Highest values are reported of biological duplicate.
* The other biological sample was < 0.05.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anifeedsci.2021.114924.

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