

# Discerning the variation in fungal-treated wheat straw as ruminant feed



NAZRI NAYAN

## Propositions

1. The effectiveness of biomass processing using different fungal strains is as varied as that of different species.  
(this thesis)
2. High potential white-rot fungal species perform well on different types of wheat straw.  
(this thesis)
3. Aesthetic judgement of data promotes good science.
4. Collaboration is great for scientists, but ethnocentrism undermines science.
5. “It is a capital mistake to theorize before one has data” – Sir Arthur Conan Doyle in *A Scandal in Bohemia* (1891); but one can always speculate.
6. On social media, the number of self-proclaimed ‘experts’ is blooming.
7. Craving for flattery is a clear sign of a lack of covert self-esteem.

Propositions belonging to the thesis, entitled:

‘Discerning the variation in fungal-treated wheat straw as ruminant feed’

**Nazri Nayan**

Wageningen, 26 October 2018

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# **Discerning the variation in fungal-treated wheat straw as ruminant feed**

Nazri Nayan

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*Untuk abah dan mak*

“

Optimism is the faith that leads to  
achievement. Nothing can be done  
without hope and confidence

”

- HELEN KELLER

# General introduction



## Towards a sustainable world

The food, feed and fuel competition – is one of the most talked about sustainability issues which sparks debate on socio-economics and environmental impact. The world average food consumption per capita is expected to increase from 2,680 kcal person<sup>-1</sup> day<sup>-1</sup> in 2015 to 3,000 kcal person<sup>-1</sup> day<sup>-1</sup> in 2030 (Bruinsma, 2003). The decrease in poverty in developing countries and the increasing demand for animal-based products, as well as a projected 30% increase in the human population by 2050, will place great pressure on food and animal production systems. Concerning “food vs. feed”, much research has been done on converting poor energy sources into more efficient and economically viable option (Banerjee, 2011). Biomass, particularly residues from agricultural harvests, are produced in abundant quantities and have great potential as valuable alternative energy sources for various applications. The worldwide production of wheat straw (*Triticum aestivum* L.), for instance, is estimated at 577 million metric tons per year, based on the assumption that about 77% of the total wheat production is straw (Ruiz *et al.*, 2012; FAO, 2016). For the next decades, biomass becomes increasingly important as a major source of bioenergy. Besides biofuels, biomass such as wheat and rice straw, has been used as a roughage source for animals by farmers, especially in the developing countries (Sarnklong *et al.*, 2010). However, this biomass has a low feeding value compared to other conventional roughage sources. Meanwhile, stubble burning after a crop harvest is still being practiced in some parts of the world, such as India, which negatively impacts global air quality (Mittal *et al.*, 2009). This stresses the importance for a more efficient use of this biomass by processing it into higher value products.

## The lignocellulosic biomass

The main components of lignocellulose are cellulose, hemicellulose and lignin which account for ~86% of the dry matter. When analyzed in the same laboratory, wheat straw for instance, contains (on dry matter basis) 46 – 50% cellulose, 26 – 31% hemicellulose and 7 – 9% lignin (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015a). It also contains pectin and other minor components such as protein, lipid and low molecular weight carbohydrates (Chen, 2014). The structural proteins are usually classified as hydroxyproline-rich glycoproteins (HRGPs), arabinogalactan proteins (AGPs), glycine-rich proteins (GRPs) and proline-rich proteins (PRPs) (Kubicek, 2013). These components are widely distributed throughout the plant structures, which depends on the anatomical parts and stage of the cell differentiation (Hansen *et al.*, 2013; Moore and Jung, 2001). Two main parts that constitute the wheat straw are leaves and stems, which make up 25 – 50 and 45

– 70%, respectively, of the total straw weight (Zhang *et al.*, 2014). The leaves contain mainly parenchyma cells, which are composed of only primary cell walls, but without highly-lignified secondary cell walls; whereas, the vascular bundles (xylem and phloem) in stems are supported by thick sclerified parenchyma cells, which contain also lignified secondary walls (Hansen *et al.*, 2013; Zhang *et al.*, 2014). Both parts are also coated with a dense epicuticular wax-rich epidermis. All these factors – leaf to stem ratio, wax coating, arrangement and density of the vascular bundles, amount of sclereids, degree of lignification and also the structural complexity of the cell wall constituents, lead to the recalcitrance and heterogenic nature of the biomass (Himmel *et al.*, 2007; Moore and Jung, 2001).

### Valorization of biomass for ruminant and its challenges

One of the main challenges to use biomass as a ruminant feed is to overcome the lignin barrier. Lignin is a vital component of the plant cell wall that provides structural strength and rigidity, limits water permeability and impedes pathogenic organisms (Moore and Jung, 2001). Lignin is deposited at a later stage of cell maturation and cross-links with other cell wall polymers, particularly to arabinoxylans via ether and ester-linked hydroxycinnamic acids (Buranov and Mazza, 2008). The composition of the cell wall components with respect to lignification is highly variable and influenced by various extrinsic and intrinsic factors – among others, different genotypes and physiological responses to environmental stimuli (Moore and Jung, 2001). In ruminant nutrition, lignin is generally regarded as an anti-quality factor in forages. In the rumen, structural polysaccharides can be degraded by fibrolytic microbes, such as *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Krause *et al.*, 2003). The degradation of lignin by the rumen microbes, however, has not been completely resolved. A monoculture of the rumen fungus, *Neocallimastix patriciarum* has been shown to solubilize 34% of the lignin extracted from pre-digested sorghum stem (McSweeney *et al.*, 1994). In the same study, they also showed the inability of the fungus to break ether linkages between lignin and polysaccharides, and interunit linkages of the lignin model compounds. The *in vivo* degradation of lignocellulose by rumen fungi is rather complex due to their slower growth rates and interactions with other rumen microbes. In addition, lignin has an inhibitory effect on other polysaccharide degrading enzyme productions (Lowe *et al.*, 1987). The loss of lignin reported in an *in vitro* study is likely due to the solubilization from the plant cell wall (Akin and Benner, 1988). Therefore, in general, the encrustation of lignin in the cell wall matrix presents a great

challenge for an efficient utilization of the cell wall polysaccharides by the rumen microbes, thus making biomass with higher lignin content a poorly digestible feed source. Besides lignin, other factors such as cellulose crystallinity, degree of polymerization and surface area can also limit an efficient utilization (Hendriks and Zeeman, 2009).

A crucial step in optimizing the utilization of biomass for ruminants is to make the polysaccharides more accessible for the carbohydrate lysing enzymes produced by the rumen microbes. For this purpose, there are various pretreatment methods available including physical and chemical approaches (Chaturvedi and Verma, 2013; Hendriks and Zeeman, 2009; Sarnklong *et al.*, 2010). Physical processing can be performed by milling, irradiation, steam pretreatment/steam explosion (ST/SE) and hydrothermal treatments. Using thermal treatments, the biomass can be heated up to 150 – 180°C, which will solubilize hemicellulose and later lignin. The biomass can also be processed chemically through acid or alkaline pretreatments. Acid hydrolysis, for instance, results in a high recovery of hemicellulosic sugars in the liquid fraction and an enhanced digestibility of cellulose in the solid fraction (Jönsson and Martín, 2016). A physicochemical treatment combination such as Ammonia Fiber Explosion (AFEX) also offers a great potential as a pretreatment method. Using AFEX, biomass is treated with liquid anhydrous ammonia at moderate temperatures (60 – 100°C) and high pressure (250 – 300 psi), before a rapid release of pressure (Teymouri *et al.*, 2005). All these pretreatment methods are able to increase the cellulose accessibility by decreasing its crystallinity and increasing the extent of delignification in a relatively short time, which makes them promising for industry-scale applications (Hendriks and Zeeman, 2009; Isroi *et al.*, 2011). Some disadvantages of these physicochemical methods include concerns regarding their environmental impact, as well as significant production costs, which makes these various pretreatment methods unsuitable for small-scale farmers' application. In addition, there is a risk that the formation of volatile products can inhibit anaerobic fermentation (Hendriks and Zeeman, 2009; Jönsson and Martín, 2016). Examples are furan aldehydes and hydroxymethylfurfural (HMF) that can be formed when treating biomass at a temperature higher than 160°C, especially in an acidic environment; while acid hydrolysis may lead to an increase in formic and levulinic acids from sugar degradation. These compounds may inhibit microbial activity and hence, lower the fermentation of carbohydrates in the rumen.

There are at least 26 review papers (in my collection) that discuss the available processing methods to transform biomass into various value-added products and for numerous applications. Sixteen of these review papers also acknowledge the great potential of using fungi in the valorization of biomass for various purposes. The extensive discussion on this topic in the scientific domain, especially since the last 20 years, is evidence of a keen interest to bring this eco-friendly approach into practice. The prospect of fungi as an alternative processing method of biomass into ruminant feed has received great attention in recent years (Shrivastava *et al.*, 2014; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2017). White-rot fungi are the most studied group of wood decaying fungi – the others being brown-rot and soft-rot fungi. Nearly all of these fungi are members of the class basidiomycetes, although some ascomycetes that cause pseudo-white rot are also classified into this group (Hatakka and Hammel, 2010). White-rot fungi are the only fungi that are capable of depolymerizing lignin completely by cleaving the C $\alpha$ -C $\beta$  bond in its substructures (Kubicek, 2013). In comparison, brown-rot fungi act primarily on cellulose and hemicellulose but they are not able to completely depolymerize lignin although some modification through demethylation may occur. The soft-rot mechanisms are characterized by an extensive loss of structural carbohydrates and the fungi do not affect lignin (Hatakka and Hammel, 2010; Kubicek, 2013).

Although white-rot fungi possess a great potential in the bioprocessing of biomass, there are numerous challenges and issues that still need to be addressed to make this method feasible for industrial application, which have recently been reviewed (Van Kuijk *et al.*, 2015a). Among factors that influence the effectiveness of the pretreatment are fungal species/strains, substrates used, culture conditions and colonization time. The relatively longer pretreatment time is one of the biggest shortcomings in using this biological approach, which may take weeks compared to just several minutes, for instance, when using AFEX (Teymouri *et al.*, 2005; Van Kuijk *et al.*, 2015b). A period of at least 4 to 8 weeks is typically needed to have a significant improvement in the ruminal degradability of the treated substrates (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015b). Research to optimize fungal treatment of biomass has been carried out, which included finding the best fungi-substrate combinations (Tuyen *et al.*, 2013), varying particle size of the substrate (Van Kuijk *et al.*, 2016) and supplementing the substrate with additives such as manganese and linoleic acid (Van Kuijk *et al.*, 2015b). Another uncertainty using fungal pretreatment is the extent of carbohydrate loss in terms of relative enrichment of the biomass and also possible release of inhibitory by-products.



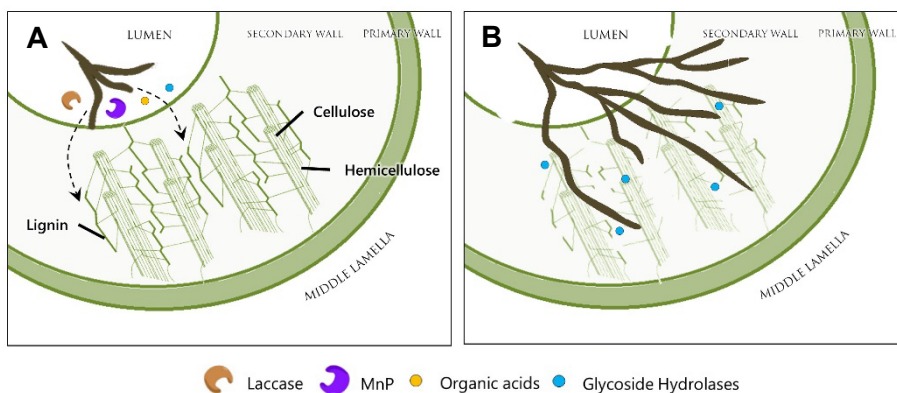
## Variation in the fungal pretreatment

The usual classification of wood-decaying fungi into white-rot, brown rot and soft rot does not necessarily justify the variation in mechanisms of degradation of lignocellulose found in the basidiomycetes. An analysis of 33 genomes of basidiomycetes revealed that some species lack lignin-degrading peroxidases (PODs), which resemble brown-rot fungi, but possess the cellulose-degrading traits typical for white-rot fungi (Riley *et al.*, 2014). Therefore, selecting the right fungal species (and strains; see definition in Textbox 1.1) is an integral element in advancing the potential in improving the nutritive value of biomass for ruminants. White-rot fungi can be further characterized based on their ability to degrade different constituents of the cell wall, which is selective or non-selective delignification (Blanchette, 1995; Martinez *et al.*, 2005). Figure 1.1 illustrates general mechanisms of fungal attack on lignocellulosic biomass. A non-selective fungus colonizes progressively from the lumen and the growing tip of the hyphae fill the eroded zones. This type of fungus causes a simultaneous loss of lignin and polysaccharides. On the other hand, some species preferentially degrade lignin and cause a minimum loss of the polysaccharides. This type of fungi are able to degrade lignin with less protruded hyphae in the secondary wall and middle lamella matrices through diffusion mechanisms (small radicals chelated with organic acids or low molecular weight mediators) (Hatakka and Hammel, 2010). These classifications are not exclusive, whereby, a particular fungus may be selective on a substrate, but show an opposite characteristic when grown on a different substrate. Some fungi can even show both characteristics on the same substrate which was observed for wood decay by *Ganoderma* spp. (Adaskaveg *et al.*, 1990; Adaskaveg and Gilbertson, 1986).

### Textbox 1.1. Definition of 'species' and 'strain'

Throughout the thesis, the terms 'species' and 'strain' are frequently used. A fungal **species** is defined as a group of related organisms that are capable of exchanging genes and interbreeding, which is designated by a Latin binomial, e.g. *Ceriporiopsis subvermispora*. A **strain** is a sub-variety of a particular fungal species that has clear-cut phenotypic and/or genetic characteristics. Therefore, the white-rot fungi species *Ceriporiopsis subvermispora* have multiple strains that can be characteristically different from each other.

Fungi degrade the cell wall components by secreting a complex mixture of extracellular enzymes as their main mode of action (Dashtban *et al.*, 2010; Hatakka and Hammel, 2010). The major lignin-degrading enzymes that are produced by white-rot fungi are class II peroxidases, such as lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidases (VP) as well as phenol oxidase, such as laccase (Kirk and Farrell, 1987; Pollegioni *et al.*, 2015). The LiP, MnP and VP are heme-containing peroxidases that act on the lignin through the formation of intermediate radicals in multiple electron transfer steps (Hatakka and Hammel, 2010). Together with laccase, these peroxidases can act on both phenolic (< 20% of lignin) and non-phenolic (> 80%) lignin substructures, with or without the presence of mediators. LiP is a mediator-free peroxidase that can degrade both phenolic and non-phenolic lignin by various structural modification, i.e. C $\alpha$ -C $\beta$  cleavage, demethylation and oxidation of benzylic methyl groups (Asina *et al.*, 2017). MnP preferentially oxidizes Mn<sup>2+</sup> into reactive Mn<sup>3+</sup>, which in its chelated form (with carboxylic acids) can only attack phenolic lignin (Hofrichter, 2002; Pollegioni *et al.*, 2015). MnP forms reactive radicals by oxidizing mediators, such as glutathione as well as unsaturated fatty acids, which can also attack nonphenolic lignin (Hofrichter, 2002). VP has a broad substrate preference and shows typical features of both LiP and MnP (Pollegioni *et al.*, 2015). Although laccase has broader functions in fungi, it



**Fig. 1.1.** Schematic representation of a fungal attack on lignocellulosic biomass. Fungal hyphae grow progressively from the lumen into the secondary cell wall (A to B). Depending on the species, various extracellular enzymes are excreted to degrade lignin (e.g. manganese peroxidase (MnP) and laccase) and also hydrolyze polysaccharides (glycoside hydrolases, GHs). A selective fungus causes less erosion that furrowed with hyphal growth, but rather degrades the cell wall constituents through diffusion mechanisms using various chelating agents or low molecular weight mediators.

can also oxidize both phenolic and non-phenolic lignin where its extensive mediator system has been reviewed (Christopher *et al.*, 2014). It has been suggested that laccase (and also MnP) attacks the phenolic lignin first, before degrading the more recalcitrant non-phenolic structure (Camarero *et al.*, 1994; Christopher *et al.*, 2014). The complete mineralization of lignin into water-soluble compounds and CO<sub>2</sub> by these enzymes is a highly oxidative process, which explains the limited degradability in the rumen environment. The extent of this enzymatic degradation can be species-dependent and variable under different culture conditions, such as varying pH and nitrogen sources (Boyle *et al.*, 1992).

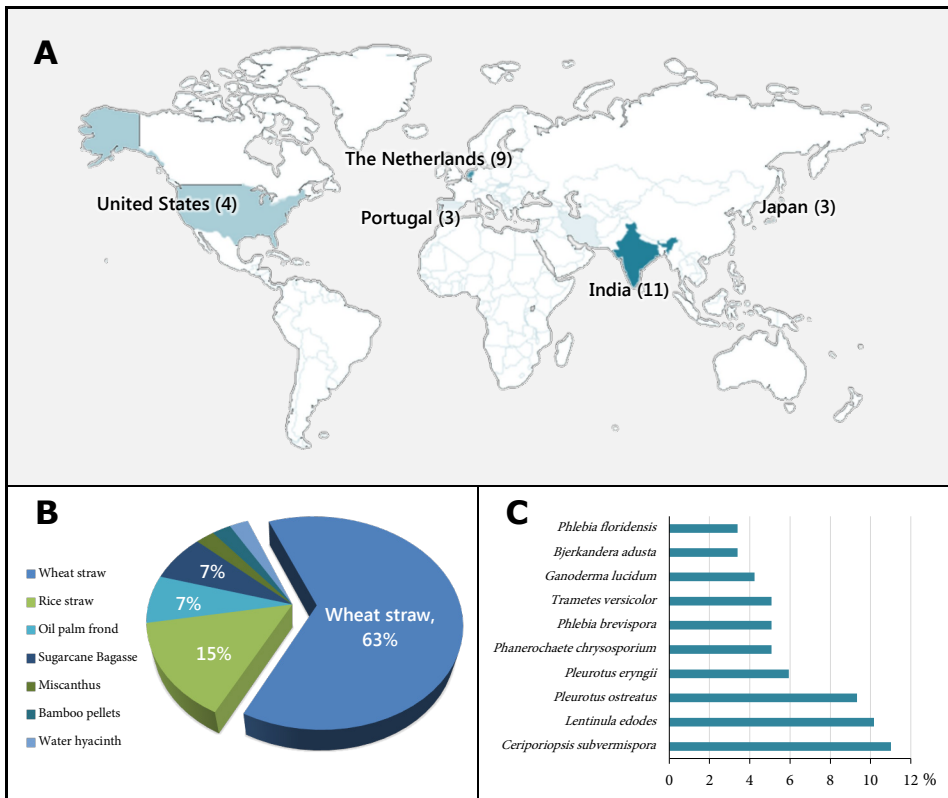
The availability of nutrients, particularly carbon and nitrogen sources have great effects on the activity of lignin-degrading enzymes, which contribute to the variation in the effectiveness of fungal pretreatment. Nutrient manipulation of the growth medium, especially nitrogen, will affect the level of enzyme production by the fungi. *Phanerochaete chrysosporium* showed a decrease in its lignin-degrading enzyme activity by a high nitrogen supplementation, in contrast to a high activity in *Pleurotus ostreatus* (Commanday and Macy, 1985; Leatham and Kent Kirk, 1983). The selective delignification of *Bjerkandera* sp. was improved by removing nitrogen from hemp stemwood (Dorado *et al.*, 2001). Nitrogen seems also to inhibit the delignification of <sup>14</sup>C-labelled wood lignin by *Lentinula edodes*, *P. chrysosporium* and *Schizophyllum commune* (Boyle *et al.*, 1992). Lignin itself is not a growth substrate for fungi (Kirk and Farrell, 1987) – these are the cell wall polysaccharides. Thus, fungi also secrete glycoside hydrolases, the most diverse group of enzymes that cleave the glycosidic bonds of cellulose and hemicellulose (Kubicek, 2013). Among the important enzymes that hydrolyze cellulose are endo- $\beta$ -1,4-glucanases, exo- $\beta$ -1,4-glucanases and  $\beta$ -glucosidases; while the hydrolysis of hemicellulose involves a wide variety of enzymes due to the nature of the polymer (Jovanovic *et al.*, 2009). These enzymes work synergistically in degrading the cell wall polysaccharides and are vital for fungal growth and metabolism (Wan and Li, 2012). As such, fungal pretreatment results in an inevitable loss of the valuable carbohydrates. With a great variety of fungal species (and strains) and also the heterogeneity of the lignocellulosic biomass available, a variable success in improving the nutritive value of the biomass can be expected, which has been extensively reviewed (Van Kuijk *et al.*, 2015b).

## The fungi and model substrates

A simple meta-analysis was performed on 37 studies that used white-rot fungi to treat various types of biomass, with specific focus to bioprocess them into animal feed (Fig. 1.2). These studies were published between 1982 and January 2018 in peer-reviewed journals. Of these studies, 63% used wheat straw as the model substrate, while rice straw (15%), oil palm fronds (7%) and sugarcane bagasse (7%) were among other popular choices. There were 44 different fungal species tested with only 3 studies including different strains of a particular species as part of their treatments (Fazaeli *et al.*, 2004; Okano *et al.*, 2006; Rodrigues *et al.*, 2008). Since 2006, *C. subvermispota* is the most studied species (11%) for bioprocessing of biomass into ruminant feed, followed by *L. edodes* (10.2%) and *P. ostreatus* (9.3%); *P. eryngii* is at the fourth place. *C. subvermispota* showed the highest potential in improving the ruminal degradability of wheat straw (17.4 – 57.5%; n = 5) with a high delignification capability (25.2 – 76.9%; n = 5). The mean improvement of ruminal degradability by *C. subvermispota* and *L. edodes* were relatively comparable when grown on rice straw (18.7 vs. 12.6% for *C. subvermispota* and *L. edodes*, respectively; n = 2), oil palm fronds (105.6 vs 96.0%; n = 2) and sugarcane bagasse (40.6 vs 47.6%; n = 2). *P. eryngii* caused a considerable increase in ruminal degradability on oil palm fronds (74%; n = 2), wheat straw (21.4%; n = 3) and rice straw (4.2%; n = 2), but not on sugarcane bagasse where a 3.8% (n = 2) decrease was observed. Although a larger set of studies may be required to be more conclusive, this pilot meta-analysis reveals a significant variation in fungal pretreatment of different biomasses, and even on the same type of biomass (Jalč *et al.*, 1998; Rahman *et al.*, 2011; Tuyen *et al.*, 2012).

Many of these studies have reported considerable successes in the bioprocessing of biomass into value-added products using fungi for various purposes, including animal feed (Sharma and Arora, 2010; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015b). Different biomasses that are used as the growth substrates, produce variable effects on the effectiveness of a particular fungal species. For instance, *C. subvermispota* degraded 16% of the lignin in maize stover, but showed a higher delignification capability on rice straw, oil palm frond and sugarcane bagasse, where more than half of the lignin contents were degraded (Tuyen *et al.*, 2013). In the latter study, the lignin content of the untreated maize stover (7.8%) was comparable to that of rice straw (6.2%), while lignin contents of oil palm fronds and sugarcane bagasse were 16.7 and 11.4%, respectively. This study indicates the importance to screen different fungal species/strains using the same substrate; and later, reproduce the same selection trial on other biomasses. A considerable variation is also seen when different species were used to treat the same biomass, i.e. wheat straw (Table

1.1). Tuyen *et al.* (2012) tested the capabilities of 11 different white-rot fungi species in improving the ruminal degradability of wheat straw – only 3 species showed significant potential: *C. subvermispora*, *L. edodes* and *P. eryngii*. Different strains of each of these 3 species were subjected to the selection trial of this thesis; and wheat straw was used as the model substrate.



**Fig. 1.2.** Summary of 37 scientific articles dealing with bioprocessing of biomass into animal feed using white-rot fungi. A: Heat map showing locations of the principal research groups with a darker shade indicating a higher number of publications. Selected countries are indicated with the number of papers in parentheses; B: Types of biomass used. Wood chips (used in 6 studies) was excluded from the analysis; C: Top 10 choices of fungal species (out of 44 different species).

**Table 1.1.** Degradation of the cell wall components and changes in the *in vitro* degradability of wheat straw treated with various white-rot fungi species.

Fungi	Time (days) <sup>a</sup>	Composition of untreated straw (%) <sup>b</sup>			Degradation (%)			Changes in deg. (%) <sup>c</sup>	Ref.
		Cell	Hcell	ADL	Cell	Hcell	ADL		
<i>C. subvermispora</i>	56	44.0	32.3	7.5	-11.4	73.1	77.3	+34.6	[8]
<i>C. subvermispora</i>	49	46.1	28.9	9.2	-5.0	53.6	59.8	+57.5	[7]
<i>Crinipellis sp.</i>	15	39.5	29.4	10.5	15.9	48.3	27.6	+10.3	[5]
<i>L. edodes</i>	84	48.0	26.0	8.1	-5.6	34.2	45.7	+23.1	[9]
<i>L. edodes</i>	56	44.0	32.3	7.5	-18.0	58.7	61.3	+27.7	[8]
<i>L. edodes</i>	49	46.1	28.9	9.2	-3.9	46.0	52.2	+35.0	[7]
<i>L. ulmadum</i>	30	41.2	25.0	8.9	-1.9	15.2	10.1	-18.2	[4]
<i>P. eryngii</i>	21	54.8	20.7	8.0	8.2	10.6	10.0	+33.8	[3]
<i>P. ostreatus</i>	30	34.1	30.8	9.8	37.8	45.1	37.8	-8.8	[6]
<i>P. ostreatus</i>	120	45.3	26.3	10.9	24.7	71.9	34.9	n/d	[1]
<i>P. ostreatus</i>	30	41.2	25.0	8.9	5.1	26.4	30.3	+22.7	[4]
<i>P. sajor-caju</i>	20	30.8	25.8	13.6	55.8	48.1	52.9	+35.3	[2]
<i>P. eryngii</i>	49	46.1	28.9	9.2	-1.7	22.5	27.2	+25.0	[7]
<i>P. ostreatus</i>	30	41.2	25.0	8.9	12.4	40.4	15.7	+15.9	[4]
<i>T. gibbosa</i>	30	41.2	25.0	8.9	7.5	62.4	27.0	+29.5	[4]
<i>T. versicolor</i>	30	34.1	30.8	9.8	34.0	39.9	31.6	+1.4	[6]

ADL: acid detergent lignin; Cell: cellulose; Hcell: hemicellulose; n/d: not determined; References: [1] Adamović *et al.*, 1998; [2] Bisaria *et al.*, 1997; [3] Fazaeli *et al.*, 2004; [4] Jalč *et al.*, 1998; [5] Shrivastava *et al.*, 2014; [6] Shrivastava *et al.*, 2011; [7] Tuyen *et al.*, 2012; [8] Van Kuijk *et al.*, 2017; [9] Van Kuijk *et al.*, 2015a.

<sup>a</sup> colonization time.

<sup>b</sup> determined by Van Soest *et al.* (1991) method.

<sup>c</sup> measure of ruminal degradability (*in vitro* gas production or organic matter digestibility).

## Research justification

This research was part of the “More Meat and Milk from Straw” project (*see* Textbox 1.2). The unique capability of fungi during their vegetative growth stage is being exploited in this project to improve the degradability of wheat straw, which has a relatively poor nutritional value for ruminants. The outcome of this thesis will contribute to a better understanding of variation among different fungal strains in changing the nutrient composition and structural characteristics of the wheat straw. Furthermore, these phenotypic variations will benefit fungal breeder in selecting strains with the desired traits, to produce a superior strain with a better bioprocess capability.

### Textbox 1.2. “More Meat and Milk from Straw” Project

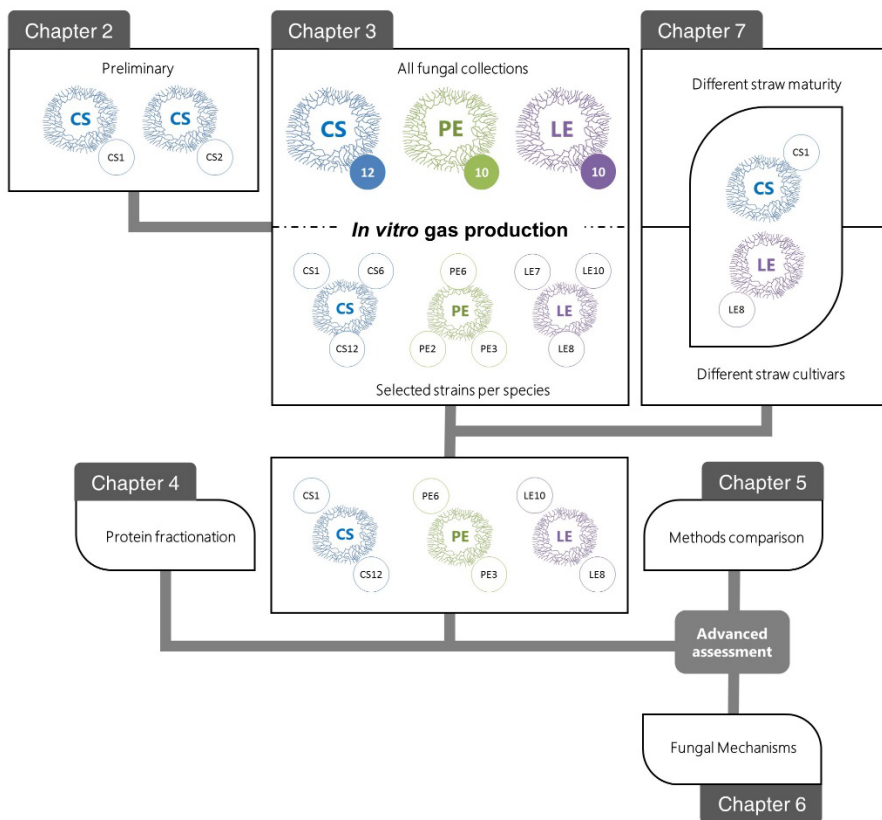
A partnership project between the Animal Nutrition and Mushroom Research Groups of Wageningen University, the Netherlands and funded by the Victam Foundation, Deka and Agrifirm. The project encompasses 3 main aspects, which are translated into 3 different PhD sub-projects: (1) Selecting the best performing fungal strains (*this thesis*); (2) Possibility to ensile the fungal-treated straw; (3) Production performance of animals fed with rations containing fungal treated straw. These sub-projects have the common aim: increasing the efficiency of ruminants in utilizing poorly digestible biomass by an optimized method of fungal pretreatment, i.e. using high potential fungal strains.

This eco-friendly approach has the potential to make a significant contribution to the global feed and food supply, to meet the increasing demand for animal-based products of the ever growing human population, in a sustainable manner.

#### Aim and thesis outline

The general aim of this thesis is to discern the biological variation among genetically different fungi in the bioprocessing of biomass into ruminant feed and selecting the best performing fungal strains, by using wheat straw as the model substrate. The outline of this thesis is illustrated in Fig. 1.2. As a start to the screening trial, in **Chapter 2**, two strains of *C. subvermispora* were assessed for their capabilities to improve the *in vitro* degradability in the rumen, as measured using a gas production technique (Cone *et al.*, 1996) in relation to their growth characteristics and lignin-degrading enzyme activities. In the next stage (**Chapter 3**), different strains from 3 promising fungal species (Tuyen *et al.*, 2012): *C. subvermispora*, *P. eryngii* and *L. edodes* were assessed for their growth characteristics and the screening of the best performing fungal strains was investigated using *in vitro* degradability in rumen fluid as a selection criterion. In the same chapter, three of the best strains for each species were further compared for their differences in gas production kinetics and other fermentation characteristics; as well as changes in the mass balance of the wheat straw. Two high-ranked strains of each species were subjected to three follow-up studies involving variation in protein solubilization (Chapter 4) and advanced assessment of the fungal-treated wheat straw (Chapter 5 and 6). In **Chapter 4**, the crude protein of the fungal-treated wheat straw was fractionated according to Sniffen *et al.* (1992); and the substrate was also evaluated for *in vitro* protein fermentation (Cone

*et al.*, 2009). In **Chapter 5**, accuracy in the assessment of cell wall components in the fungal-treated straw using the standard gravimetric method was compared to the more specific pyrolysis-GC/MS and monosaccharide analysis methods, and their relationship to the *in vitro* gas production. **Chapter 6** characterized the unique mechanisms of different fungal species in degrading lignin using a combined pyrolysis-GC/MS and 2D-nuclear magnetic resonance (NMR) approach. The persistency of the selected high potential strains of *C. subvermispora* and *L. edodes* on improving wheat straw of different maturities and cultivars were studied in **Chapter 7**. Finally, the findings from all 6 research chapters are discussed in **Chapter 8**.



**Fig. 1.3.** The general outline of this thesis. CS: *Ceriporiopsis subvermispora*; PE: *Pleurotus eryngii*; LE: *Lentinula edodes*. Shaded circles indicate number of strains used, while selected strain numbers are shown in white circles.



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“

We need an opposition to remind us if we are making mistakes. When you are not opposed you think everything you do is right.

”

- MAHATHIR MOHAMAD

# Differences between two strains of *Ceriporiopsis subvermispora* on improving the nutritive value of wheat straw for ruminants

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## ABSTRACT

The present study evaluated differences between two strains of *Ceriporiopsis subvermispota* on improving the nutritive value and *in vitro* degradability of wheat straw. Wheat straw was treated with the fungi for 7 weeks. Weekly samples were analyzed for ergosterol content, *in vitro* gas production (IVGP), chemical composition and lignin degrading enzyme activity. Ergosterol data showed CS1 to have a faster initial growth than CS2 and reaching a stationary phase after 3 weeks. The IVGP of CS1-treated wheat straw exceeded the control earlier than CS2 (4 vs 5 weeks). CS1 showed a significantly higher ( $P < 0.001$ ) selectivity in lignin degradation compared to CS2. Both strains showed peak activity of laccase and manganese peroxidase (MnP) at week 1. CS1 showed a significantly higher ( $P < 0.001$ ) laccase activity, but lower ( $P = 0.008$ ) MnP activity compared to CS2. Both CS strains improved the nutritive value of wheat straw. Variation between strains was clearly demonstrated by their growth pattern and enzyme activities. The differences among the two strains provide an opportunity for future selection and breeding programs in improving the extent and selectivity of lignin degradation in agricultural biomass.

**Keywords:** *Ceriporiopsis subvermispota*; strains differences; lignin degradation; *in vitro* gas production; enzymes; wheat straw; ruminants



## INTRODUCTION

Agricultural biomass, such as wheat straw has great potential as a valuable resource for a wide range of applications, including utilization as an animal feed. Many of these agricultural by-products are available in abundant quantities (Isroi *et al.*, 2011). Utilization of wheat straw as a feed ingredient by ruminants is considered to be relatively low – 51% digestible organic matter *in vivo* (Lesoing *et al.*, 1980), due to encrustation with lignin that hinders the degradation of cellulose and hemicellulose by the rumen microbes (Van Kuijk *et al.*, 2015a). Therefore, complete or partial removal of lignin in the wheat straw is a prerequisite to realize its potential as a ruminant feed.

Several physical and chemical methods have been used to overcome the lignin barrier in the utilization of lignin-rich biomass (Hendriks and Zeeman, 2009). These methods, however, are often associated with significant costs and/or produce waste that can be detrimental to the environment (Isroi *et al.*, 2011). Biological methods, including the use of fungi have many advantages over chemical and physical methods (Dinis *et al.*, 2009; Shrivastava *et al.*, 2011; Tuyen *et al.*, 2012). Among the white-rot fungi, *Ceriporiopsis subvermispora* (Gilbertson & Ryvarden, 1985), has been shown to have unique abilities to selectively degrade lignin and increase the fermentability of treated biomass by the rumen microflora (Shrivastava *et al.*, 2011; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015a). *C. subvermispora* has been studied in various other applications such as biopulping (Aguiar *et al.*, 2010; de Souza-Cruz *et al.*, 2004), enzymatic hydrolysis and bioethanol production (Wan and Li, 2010).

Degradation of lignin by white-rot fungi is a multi-enzymatic process with four major lignin degrading oxidases being involved with a number of auxiliary enzymes (Pollegioni *et al.*, 2015). Lignin peroxidase (LiP) is an important enzyme in fungal ligninolysis, due to its very high redox potential, allowing the oxidation of recalcitrant non-phenolic lignin substructures (> 80% of lignin) (Hatakka and Hammel, 2010; Pollegioni *et al.*, 2015). Manganese peroxidase (MnP) is the most common peroxidase and is secreted by many white-rot fungi. This heme enzyme catalyzes the oxidation of  $Mn^{2+}$  into  $Mn^{3+}$ . The latter is chelated by organic acids and acts as a diffusible oxidant of phenolic lignin substructures (< 20% of lignin) (Kirk and Farrell, 1987). Versatile peroxidase (VP) has a broad substrate specificity and shares the typical features of LiP and MnP. Laccase is a copper-containing enzyme which has a wide range of functions in fungi, including the formation of fruiting bodies, pigmentation, stress defense, as well as lignin degradation. The role of laccase in lignin degradation has been the subject of interest in numerous studies (Arora *et al.*, 2002; Arantes and Milagres, 2007; Dinis *et al.*, 2009).

Assessment of enzyme activities will provide a better understanding on the selectivity of different fungal species/strains in degrading lignin. It can be an important criterion for selecting a potential fungus for breeding programs. Different white-rot fungi species showed different effects on the lignocellulose composition and ruminal degradability of the treated substrate (Tuyen *et al.*, 2012). In addition, Mata *et al.* (1998) observed quantitative variations in the enzyme activities of six strains of *Lentinula edodes* grown on wheat straw, although they exhibited a similar pattern along the colonization weeks. These studies indicate that not only examining fungi-biomass combination is important but also screening strains within species to optimize the selective lignin degradation of lignin-rich biomass.

As a start for future selection of the best performing fungal species and strains, a study was conducted with the aim to evaluate differences between two strains of *C. subvermispora* for their ability to improve the *in vitro* degradability and nutritive value of the wheat straw in the rumen in relation to their growth characteristics and lignin-degrading enzymes activities.

## MATERIALS AND METHODS

### Preparation of fungal strains and spawn

Two strains of *C. subvermispora*, CS1 (MES 13094/CBS 347.63) and CS2 (MES 14407/ACCC31512) maintained in the liquid nitrogen collection of the Plant Breeding group, Wageningen, the Netherlands were used. Both strains were propagated on malt agar extract, containing malt extract 20.0 g l<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 0.5 g l<sup>-1</sup>, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g l<sup>-1</sup>, Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O 0.5 g l<sup>-1</sup> with a pH of 5.4 and incubated at 24°C until the mycelia colonized most of the agar surface. The spawns of CS1 and CS2 were prepared by placing a piece of the agar culture (1.5 × 2.0 cm) into a container containing sterilized sorghum grains and incubated at 24°C for 4 to 5 weeks. The sorghum grains were sterilized by submerging in boiled water, then drained for the excess water and autoclaved at 121°C for 20 min.

### Substrate preparation, fungal inoculation and sampling

Wheat straw (*Triticum aestivum* L.) was used as substrate which was chopped into approximately 3 cm pieces and soaked in tap water for 3 days at room temperature. Water was drained from the wet straw through a perforated strainer (pore size = 5 mm) for 5 hours resulting in a final moisture content of 76.8%. The straw was then distributed into 172×110×70 mm (1200 ml) micropropagation containers (Combiness, Nevele, Belgium)

with approximately 160 g per container. All containers were autoclaved at 121°C for 1 h and allowed to cool overnight in an aseptic room at room temperature. Pre- and post-autoclaved wheat straw was sampled to determine the effect of autoclaving on the chemical composition. The autoclaved straw was aseptically inoculated with the spawn at 10% of the estimated dry weight. The containers containing inoculated straw, along with containers of only autoclaved straw (control) were incubated in triplicate at 24°C for 7 weeks in a climate controlled chamber. Treatments (control, CS1 and CS2) were randomized in each week (block). No repeated measurement was done on the same straw sample. At each week, independent samples were weighed, thoroughly mixed and ~40 g from each container was freeze-dried for the ergosterol and enzyme assays. The remaining sample from the same container (~120 g) was air-dried at 70°C for a week and ground over a 1 mm sieve using a cross beater mill (100AN, Peppink, Olst, the Netherlands) for chemical analyses and *in vitro* gas production.

### Estimation of fungal biomass

Fungal biomass was estimated by an ergosterol assay as described by Niemenmaa *et al.* (2008). In brief, a freeze-dried sample (~200 mg) was weighed into a glass tube and extracted with a 3 ml 10% potassium hydroxide in methanol solution on a rotary shaker (Labotech, Maharashtra, India) for 10 min at 230 rev min<sup>-1</sup> before saponification at 80°C in a heating block for 60 min. The ergosterol was extracted by adding 1 ml distilled water and 2 ml hexane to the tube. The hexane phase was dried in a vacuum evaporation system (Rapidvap, Kansas, MO, USA) before being re-dissolved in methanol. The solution was filtered into a HPLC vial for Waters HPLC-PDA analysis (Alliance HPLC system, Milford, USA). A reversed phase C18 column (250×4.6 mm, Phenomex aqua 5 µm) was used with the liquid phase in the HPLC system being 90% methanol and 10% (1:1) 2-propanol/hexane. Cholecalciferol (vitamin D<sub>3</sub>) was used as an internal standard and the ergosterol peak was detected at 280 nm.

### *In vitro* gas production

*In vitro* gas production, expressed as ml g<sup>-1</sup> of organic matter (OM), was performed as described by Cone *et al.* (1996). In brief, rumen fluid was collected from two non-lactating, rumen fistulated cows fed 1 kg concentrate, and grass silage *ad libitum*. The strained rumen fluid was filtered through 2 layers of cheese cloth and mixed with a mineral-buffer solution. All procedures were conducted under continuous flushing with CO<sub>2</sub>. Samples were incubated with the buffered rumen fluid for 72 h and the gas

production was automatically registered. The kinetic parameters ( $A$ ,  $B$ ,  $C$ ,  $t_{Rm}$  and  $R_m$ ) were determined by fitting a biphasic model (Groot *et al.*, 1996) to the gas production data where  $A$  is the asymptotic gas production ( $\text{ml g}^{-1}$  OM) at the two different phases that represents the fermentation of soluble (phase 1) and non-soluble fractions (phase 2);  $B$  the half time of the maximum gas production (h);  $C$  is a parameter to determine the steepness of the curve;  $t_{Rm}$  time of the maximum fractional rate of substrate degradation (h); and  $R_m$  the maximum fractional rate of substrate degradation ( $\text{h}^{-1}$ ). As there was no significant change in  $B$  and  $C$  values of the first phase, only  $A_1$ ,  $A_2$  and the kinetic parameters of the second phase are presented.

### Chemical analysis

The air-dried samples were dried in an oven at  $103^\circ\text{C}$  to determine the dry matter (DM) content (ISO 6496, 1999). Ash content was determined after combustion at  $550^\circ\text{C}$  for 3 h in a muffle furnace (ISO 5984, 2002). Nitrogen content was determined by the Kjeldahl method (ISO 5983, 2005) and crude protein calculated as  $\text{N} \times 6.25$ . Neutral detergent fiber (NDF) was determined using heat-stable amylase (thermamyl) and alcalase, using the standard procedures of Van Soest *et al.* (1991). Acid detergent fiber (ADF) and acid detergent lignin (ADL) were also determined using the standard method (Van Soest *et al.*, 1991). Hemicellulose was calculated as the difference between NDF and ADF, while cellulose was calculated as the different between ADF and ADL. Absolute amounts were calculated using data on the remaining air dry matter after fungal incubation corrected for DM content in the dried materials.

### Enzyme assays

The freeze-dried samples were extracted with cold sodium acetate buffer ( $20 \text{ mmol l}^{-1}$ , pH 5.0) supplemented with 0.01% Tween 80 and 2% of polyvinylpyrrolidone (PPVP). The samples were then centrifuged at  $4500 \text{ rev min}^{-1}$  for 20 min, before filtering the supernatant through a Whatman No. 3 (Maidstone, UK) paper to obtain crude enzyme extracts. The LiP assay was based on the oxidation of Azure B ( $\epsilon_{651} = 4.88 \times 10^4 \text{ mol l}^{-1} \text{ cm}^{-1}$ ) (Archibald, 1992) with a final concentration in the mixture of  $6.4 \text{ } \mu\text{mol l}^{-1}$  in  $50 \text{ mmol l}^{-1}$  sodium tartrate buffer (pH 3) in the presence of  $0.5 \text{ mmol l}^{-1} \text{ H}_2\text{O}_2$  and measurement at 651 nm. MnP activity was determined by the oxidation of phenol red ( $\epsilon_{610} = 2.2 \times 10^4 \text{ mol l}^{-1} \text{ cm}^{-1}$ ) with addition of  $\text{MnSO}_4$  (Arora *et al.*, 2002). The final concentration of the reagents in the mixture were  $20 \text{ mmol l}^{-1}$  sodium succinate buffer (pH 4.5),  $0.5 \text{ mmol l}^{-1} \text{ MnSO}_4$ ,  $34 \text{ } \mu\text{mol l}^{-1}$  phenol red (0.01%),  $25 \text{ mmol l}^{-1}$  lactate buffer

(pH 4.5) and 0.5 mmol l<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>. The absorbance was monitored at 610 nm. The reactions in the LiP and MnP assays were initiated by the addition of H<sub>2</sub>O<sub>2</sub>. Laccase was assayed using 2,6-dimethoxyphenol (DMP) as a substrate ( $\epsilon_{468} = 4.96 \times 10^4 \text{ mol l}^{-1} \text{ cm}^{-1}$ ) (More *et al.*, 2011). The final concentration of the reagents in the mixture was 100 mmol l<sup>-1</sup> sodium acetate buffer (pH 6.0) and 1 mmol l<sup>-1</sup> DMP (Sigma-Aldrich, St. Louis, MO, USA). The reaction was monitored at 468 nm. One ml of enzyme extract was used in all three assays. The activities of the lignin degrading enzymes were calculated as follows:

$$\text{Enzyme activity (U ml}^{-1}\text{)} = \frac{\Delta OD \times V_A \times DF \times 10^6}{V_E \times \epsilon \times \Delta t}$$

where  $\Delta OD$  is the difference in absorbance ( $A_{\text{samples}} - A_{\text{blanks}}$ ) at a respective wavelength,  $V_A$  is the volume of the assay mixture (ml),  $V_E$  is the volume of the enzyme extract ( $\mu\text{L}$ ),  $DF$  is the dilution factor,  $\epsilon$  is the molar extinction coefficient and  $\Delta t$  is the time difference (min). The final enzyme activity results were expressed as U kg<sup>-1</sup> DM.

### Statistical analysis

Data were analyzed by two-way analysis of variance using the general linear model in SAS 9.3, followed by post-hoc multiple comparison using least significance differences. The statistical model used was as follows:

$$Y_{ij} = \mu + \alpha_i + \tau_j + (\alpha \times \tau)_{ij} + \epsilon_{ij}$$

where  $Y_{ij}$  = response variable  $ij$ ,  $\mu$  = overall mean,  $\alpha_i$  = the effect of treatment  $i$ ,  $\tau_j$  = effect of week  $j$ ,  $(\alpha \times \tau)_{ij}$  = interaction of treatment  $i$  and week  $j$  and  $\epsilon_{ij}$  = residual error with a mean of 0 and variance  $\sigma^2$ .  $\alpha_i$  was considered a fixed effect and  $\tau_j$  a random effect. Minimum significance threshold was set at 0.05. Pearson Product-Moment Correlation ( $r$ ) coefficients were also determined among the measured variables.

## RESULTS

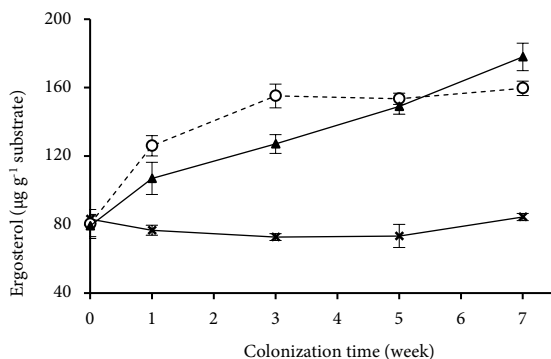
### Fungal growth

Ergosterol analyses showed that CS1 grew more rapid during the first week of colonization compared to CS2, and achieved a stationary growth phase after 3 weeks (Fig. 2.1). At week 7, CS1 had an ergosterol content of 159.6  $\mu\text{g g}^{-1}$  substrate, which was significantly lower ( $P < 0.001$ ) than CS2 which had an ergosterol content of 178  $\mu\text{g g}^{-1}$  substrate. The ergosterol content of the autoclaved straw (control) remained low and had a mean ergosterol content of 78.1  $\mu\text{g g}^{-1}$  substrate after 7 weeks.

### *In vitro* gas production

Figure 2.2 shows the cumulative IVGP after 72 h of incubation in rumen fluid of wheat straw treated with CS1 and CS2, in comparison with the autoclaved wheat straw over the 7 week period. Both fungal-treated samples showed significantly higher ( $P < 0.001$ ) IVGP after 7 weeks of colonization compared to the autoclaved wheat straw. The difference in IVGP of the 7 week CS1 and CS2-treated straw, however, was not significant. The CS1-treated straw had a significant decrease ( $P = 0.006$ ) in IVGP at week 1 compared to the corresponding straw at week 0, while CS2-treated straw continued to decrease until week 2 ( $P < 0.001$ ). Both CS-treated straw showed an increase in IVGP in the following weeks and significantly exceeded ( $P < 0.001$ ) the autoclaved straw after week 4 and 5 for CS1 and CS2, respectively.

The gas production kinetic parameters of the control, CS1 and CS2-treated straw are summarized in Table 2.1. The increase in IVGP, along the colonization weeks are reflected by the  $A_1$  and  $A_2$  values. The second phase, gas production as a result of fermentation of the non-soluble fraction, showed significant increases ( $P < 0.001$ ) in  $C_2$  and  $R_{m2}$  values during the incubation of the straw with both fungal strains. Contrast-wise, the fungal-treated straw was significantly different in the  $C_2$  ( $P = 0.025$ ) and  $R_{m2}$  ( $P = 0.002$ ) values.

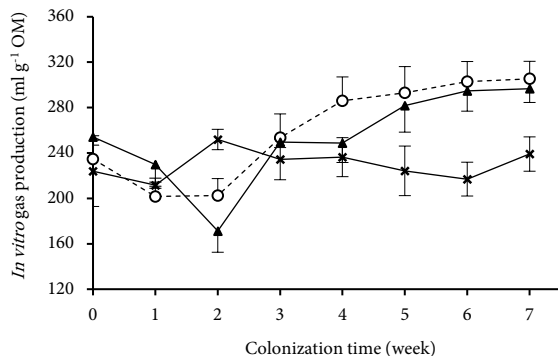


**Fig. 2.1.** Ergosterol content of autoclaved wheat straw (×) or the same straw colonized by two strains of *Ceriporiopsis subvermispora*, CS1 (○) and CS2 (▲) for up to 7 weeks. Error bars represent standard deviations.

**Table 2.1.** The effects of autoclaved wheat straw (control) treated with different *Ceriporiopsis subvermispora* strains (CS1 and CS2) at different colonization weeks on the *in vitro* gas production and its kinetic parameters at different phases.

Treatment (Trt)	Week	IVGP <sub>72</sub>	Gas production kinetic parameters					
			A <sub>1</sub>	A <sub>2</sub>	B <sub>2</sub>	C <sub>2</sub>	t <sub>Rm2</sub>	R <sub>m2</sub>
Control	0	235.4	13.5	197.5	19.34	3.00	24.37	0.08
	7	239.1	13.3	203.3	19.00	2.99	23.91	0.08
CS1	0	234.6 <sup>a</sup>	14.2 <sup>a</sup>	198.1 <sup>a</sup>	18.40 <sup>a</sup>	2.85 <sup>a</sup>	22.82 <sup>a</sup>	0.08 <sup>a</sup>
	1	201.7 <sup>b</sup>	15.2 <sup>a</sup>	160.7 <sup>b</sup>	20.26 <sup>ab</sup>	3.01 <sup>ab</sup>	25.53 <sup>ab</sup>	0.08 <sup>a</sup>
	3	253.5 <sup>a</sup>	22.2 <sup>b</sup>	201.4 <sup>a</sup>	21.52 <sup>b</sup>	3.18 <sup>bc</sup>	27.47 <sup>b</sup>	0.08 <sup>a</sup>
	5	293.1 <sup>c</sup>	33.0 <sup>c</sup>	239.3 <sup>c</sup>	20.23 <sup>ab</sup>	3.32 <sup>c</sup>	26.05 <sup>bc</sup>	0.09 <sup>b</sup>
	7	305.3 <sup>c</sup>	30.1 <sup>c</sup>	261.2 <sup>c</sup>	18.73 <sup>a</sup>	3.37 <sup>c</sup>	24.21 <sup>ab</sup>	0.10 <sup>c</sup>
CS2	0	254.1 <sup>ab</sup>	18.4 <sup>a</sup>	212.6 <sup>a</sup>	17.67	2.76 <sup>a</sup>	21.68 <sup>a</sup>	0.08 <sup>a</sup>
	1	230.0 <sup>a</sup>	21.2 <sup>a</sup>	174.1 <sup>b</sup>	19.96	2.85 <sup>a</sup>	24.78 <sup>ab</sup>	0.07 <sup>b</sup>
	3	249.5 <sup>a</sup>	21.3 <sup>a</sup>	198.3 <sup>a</sup>	19.92	2.99 <sup>ab</sup>	25.07 <sup>ab</sup>	0.08 <sup>ab</sup>
	5	281.7 <sup>bc</sup>	29.2 <sup>b</sup>	235.1 <sup>bc</sup>	19.84	3.19 <sup>bc</sup>	25.38 <sup>b</sup>	0.09 <sup>c</sup>
	7	296.6 <sup>c</sup>	31.4 <sup>b</sup>	252.8 <sup>c</sup>	19.70	3.33 <sup>c</sup>	25.39 <sup>b</sup>	0.09 <sup>d</sup>
RMSE		17.39	3.12	14.44	1.33	0.12	1.89	0.00
P value								
Trt		<0.001	<0.001	<0.001	0.122	<0.001	0.076	0.002
Time		<0.001	<0.001	<0.001	0.110	<0.001	0.14	<0.001
Trt × Time		<0.001	<0.001	<0.001	0.149	0.001	0.154	<0.001
Contrast								
Control vs Trt		<0.001	<0.001	<0.001	0.043	<0.001	0.029	0.088
CS1 vs CS2		0.209	0.581	0.152	0.898	0.025	0.671	0.002

Values with different superscripts within column of the same treatment are significantly ( $P < 0.05$ ) different. RMSE: Root mean square error; IVGP<sub>72</sub>: cumulative *in vitro* gas production at 72 h (ml g<sup>-1</sup> OM); A<sub>1</sub>, A<sub>2</sub>: asymptotic gas production (ml g<sup>-1</sup> OM) phase 1 and 2, respectively; B<sub>2</sub>: half time of the maximum gas production at phase 2 (h); C<sub>2</sub>: parameters determine the curvature of the graph; t<sub>Rm2</sub>: time of the maximum fractional rate of substrate degradation (h); R<sub>m2</sub>: maximum fractional rate of substrate degradation (h<sup>-1</sup>).



**Fig. 2.2.** Cumulative *in vitro* gas production after 72 h of incubation in rumen fluid for autoclaved wheat straw (×) or the same straw colonized by two strains of *Ceriporiopsis subvermispora*, CS1 (○) and CS2 (▲) for up to 7 weeks. Error bars represent standard deviations.

### Chemical composition

There was a significant increase ( $P < 0.012$ ) in the ADL content in the DM of the substrate as a result of autoclaving (Table 2.2). Along the colonization weeks, treatment by both *C. subvermispora* strains resulted in a significant increase ( $P < 0.05$ ) in the cellulose and CP contents; but a decrease ( $P < 0.001$ ) in the DM, NDF, ADF, ADL and hemicellulose contents (Table 2.2). The CS1 and CS2-treated straw were remarkably different in their cell wall compositions. At week 7, the cellulose content was higher ( $P < 0.001$ ) in the CS1-treated straw, but showed a lower ( $P < 0.001$ ) hemicellulose content compared to the CS2-treated straw. Difference in the ADL content between both fungal-treated substrates was not significant. The contrast between the two strains in the absolute amounts of these cell wall components, however, was only significant for cellulose.

### Enzyme activities

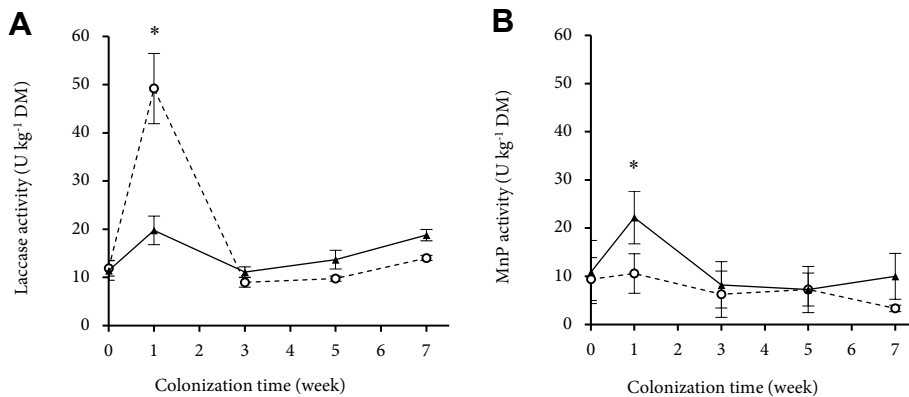
No lignin peroxidase (LiP) could be detected in any of the samples analyzed. There were significant effects ( $P < 0.001$ ) of the fungal treatment of the straw on laccase and manganese peroxidase (MnP) activity (Fig. 2.3). Both lignin degrading enzymes showed peak activities at week 1 of fungal colonization. CS1-treated straw showed a significantly ( $P < 0.001$ ) higher laccase activity at week 1 ( $49.19 \text{ U kg}^{-1} \text{ DM}$ ) compared to CS2-treated straw which showed an activity of  $19.76 \text{ U kg}^{-1} \text{ DM}$ . On the other hand, CS2-treated straw showed a MnP activity of  $22.20 \text{ U kg}^{-1} \text{ DM}$  at week 1, which was significantly ( $P = 0.008$ ) higher than the CS1-treated straw which had an activity of  $10.59 \text{ U kg}^{-1} \text{ DM}$ . After a sharp



**Table 2.2.** The effect of autoclaved wheat straw (control) treated with two different *Ceriporiopsis subvermispora* strains (CS1 and CS2) at different colonization weeks on the chemical composition and absolute amounts of nutrients.

Treatment (Trt)	Week	DM (g kg <sup>-1</sup> )	Chemical composition (g kg <sup>-1</sup> DM)				Absolute amounts (g)				
			Cell	Hcell	ADL	CP	Cell	Hcell	ADL	CP	
Untreated straw											
Pre-autoclave		187.7 <sup>a</sup>	492.9	308.6	77.7 <sup>a</sup>	27.0	24.7				
Autoclaved (control)		208.8 <sup>b</sup>	489.8	311.5	87.8 <sup>b</sup>	26.2	24.9				
CS1											
	0	207.4 <sup>a</sup>	473.4 <sup>a</sup>	299.4 <sup>a</sup>	88.2 <sup>a</sup>	28.3 <sup>ab</sup>	25.1 <sup>a</sup>		11.9 <sup>a</sup>	7.5 <sup>ab</sup>	2.2 <sup>a</sup>
	1	208.3 <sup>a</sup>	474.6 <sup>a</sup>	299.8 <sup>a</sup>	95.4 <sup>a</sup>	27.3 <sup>a</sup>	25.0 <sup>a</sup>		11.8 <sup>a</sup>	7.5 <sup>ab</sup>	2.4 <sup>a</sup>
	3	193.6 <sup>b</sup>	465.6 <sup>a</sup>	251.9 <sup>b</sup>	76.1 <sup>b</sup>	30.0 <sup>ab</sup>	25.6 <sup>a</sup>		10.9 <sup>b</sup>	5.9 <sup>c</sup>	1.8 <sup>b</sup>
	5	188.4 <sup>b</sup>	488.4 <sup>b</sup>	173.6 <sup>c</sup>	46.2 <sup>c</sup>	31.8 <sup>bc</sup>	25.2 <sup>a</sup>		10.7 <sup>b</sup>	3.8 <sup>d</sup>	1.0 <sup>c</sup>
	7	190.3 <sup>b</sup>	508.2 <sup>c</sup>	142.9 <sup>d</sup>	37.6 <sup>c</sup>	33.5 <sup>c</sup>	27.2 <sup>b</sup>		11.1 <sup>b</sup>	3.1 <sup>e</sup>	0.8 <sup>c</sup>
CS2											
	0	211.4 <sup>a</sup>	468.9 <sup>a</sup>	300.3 <sup>a</sup>	88.2 <sup>a</sup>	30.5 <sup>abc</sup>	25.7 <sup>a</sup>		11.9 <sup>a</sup>	7.6 <sup>ab</sup>	2.2 <sup>ab</sup>
	1	201.6 <sup>b</sup>	452.4 <sup>b</sup>	304.1 <sup>a</sup>	93.0 <sup>a</sup>	30.2 <sup>ab</sup>	24.0 <sup>b</sup>		10.9 <sup>b</sup>	7.4 <sup>b</sup>	2.2 <sup>a</sup>
	3	194.1 <sup>bc</sup>	447.0 <sup>b</sup>	241.0 <sup>b</sup>	86.6 <sup>a</sup>	29.4 <sup>a</sup>	26.0 <sup>a</sup>		10.4 <sup>c</sup>	5.6 <sup>c</sup>	2.0 <sup>b</sup>
	5	189.2 <sup>c</sup>	475.9 <sup>bc</sup>	183.6 <sup>c</sup>	53.0 <sup>b</sup>	33.8 <sup>bc</sup>	25.4 <sup>a</sup>		10.6 <sup>bc</sup>	4.1 <sup>d</sup>	1.2 <sup>c</sup>
	7	192.1 <sup>c</sup>	482.5 <sup>c</sup>	171.8 <sup>d</sup>	44.2 <sup>b</sup>	34.1 <sup>c</sup>	26.4 <sup>a</sup>		10.7 <sup>bc</sup>	3.8 <sup>d</sup>	1.0 <sup>c</sup>
RMSE		4.63	6.23	5.18	5.55	2.23	0.68		0.31	0.19	0.15
<i>P</i> values											
Trt		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
Time		<0.001	<0.001	<0.001	<0.001	0.097	<0.001		<0.001	<0.001	0.967
Trt × Time		<0.001	<0.001	<0.001	<0.001	0.006	0.236		0.001	<0.001	0.188
Contrast											
Control vs Trt		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001
CS1 vs CS2		0.968	<0.001	0.001	0.364	0.089	0.637		0.002	0.062	0.102

Values with different superscripts within column of the same treatment are significantly ( $P < 0.05$ ) different. RMSE: Root mean square error; DM: dry matter corrected for air-dry matter; Cell: cellulose; Hcell: hemicellulose; ADL: acid detergent lignin; CP: crude protein. Absolute amounts were calculated from the remaining air-dried materials.



**Fig. 2.3.** Laccase (A) and manganese peroxidase (MnP; B) activity in autoclaved wheat straw colonized by two *Ceriporiopsis subvermispora* strains, CS1 (O) and CS2 (▲) for up to 7 weeks. Asterisks indicate significant ( $P < 0.01$ ) difference of enzyme activity between the two strains. Error bars represent standard deviations.

decline at week 3, there was a significant increase ( $P < 0.05$ ) in laccase activity of the treated straw for both strains at week 7. No significant increase was observed in MnP activity after week 3. Contrast-wise, laccase ( $P < 0.001$ ) and MnP ( $P = 0.021$ ) activity in the CS1 and CS2-treated straw were significantly different.

## DISCUSSION

The present study investigated the *in vitro* degradability and compositional changes in wheat straw treated with two strains of *C. subvermispora* over a 7 week period. We also assessed the activities of enzymes involved in lignin degradation and the colonization of the fungi over time. From previous work in our laboratory, *C. subvermispora* (CS1; MES 13094) has been shown to be a good candidate for the bioprocessing of wheat straw and other agricultural biomass for ruminant feed (Tuyen *et al.*, 2013; Van Kuijk *et al.*, 2016b). The present study compared the performance of the previously used strain CS1 (Tuyen *et al.*, 2012) with a recently acquired strain CS2 (MES 14407). Variation within species offers opportunities to improve strain performance by breeding.

Ergosterol is regarded as a good indicator of fungal biomass, especially when the fungus is grown on solid medium (Gao *et al.*, 1993). In the present study, background ergosterol content was detected in the autoclaved wheat straw. This is not unexpected since fungal microflora of the wheat plants have been described in several reports (Larran

*et al.*, 2007; Vujanovic *et al.*, 2012). The persistence of ergosterol after sterilization has been reported by Mille-Lindblom *et al.* (2004). They found an ergosterol content of up to 250  $\mu\text{g g}^{-1}$  dry weight in common reed (*Phragmites*) litter after autoclaving, indicating that ergosterol survives sterilization. Results from the ergosterol assay showed that both *C. subvermispota* strains successfully colonized the substrate. The variation among strains of the same species is clearly demonstrated by a marked difference in the growth pattern of both *C. subvermispota* strains. The initial growth of CS1 was faster than CS2 and reached a stationary phase after 3 weeks, similar as reported for this same strain (Van Kuijk *et al.*, 2016b). CS2 on the other hand, had a slower initial colonization but sustained growth during the 7 weeks. The same growth pattern has been reported in studies on wood chips (Messner *et al.*, 1998; Niemenmaa *et al.*, 2008). Messner *et al.* (1998) for instance, observed a rapid increase in ergosterol content of *C. subvermispota* for the first 6 days of colonization, before it remained constant until day 14 with a plateau value of 45  $\mu\text{g g}^{-1}$  wood. These increases coincide with a more intensive production of aerial mycelia as the oxygen tension outside the substrate is higher allowing intensive fungal growth (Messner *et al.*, 1998). After all easily accessible resources have been used for a rapid initial growth, the rate of biomass accumulation declines as the fungus starts producing secondary metabolites (Drew and Wallis, 1983; Liao *et al.*, 1995).

*In vitro* gas production can be used as a tool to evaluate the degradability of fungal-treated wheat straw in rumen fluid. The IVGP values obtained for *C. subvermispota* by Tuyen *et al.* (2012) are comparable to the result presented here for CS1-treated wheat straw in the range of 300 to 310 ml  $\text{g}^{-1}$  OM after 7 weeks of colonization. Both fungal-treated straw samples did not differ in their IVGP values at week 7. There was also a significant improvement in the IVGP of CS1-treated straw earlier in the colonization than of the CS2-treated straw. To investigate differences in their fermentation kinetics, a simple biphasic approach was used to represent the fermentation of the soluble (phase 1) and non-soluble fractions (phase 2). The *A*, *B* and *C* values at different phases of gas production for CS1 observed by Tuyen *et al.* (2012) are almost identical to the results presented here. In the present study, higher *A*<sub>1</sub> and *A*<sub>2</sub> values with colonization week are indicative of increased amounts of fermentable soluble and non-soluble fractions. The increase in the soluble fraction is expected as fungi liberate free sugars during their colonization. Free sugars were not measured in the present study, but a three-fold increase in the amount of total non-structural carbohydrates have been reported in fungal-treated rice straw (Karunanandaa and Varga, 1996). Phase 2 is related to the fermentation of structural (non-soluble) carbohydrates. With increasing colonization time, the gas

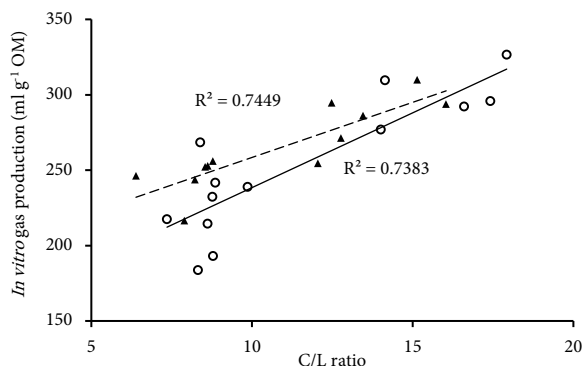
production profiles became more sigmoidal with an increasing slope, as indicated by the higher  $C_2$  values. Significant contrasts between CS1 and CS2-treated straw in their  $C_2$  and  $R_{m2}$  values show that CS1 has a slight advantage over CS2 in improving the fermentation of structural carbohydrates.

The autoclaving process significantly affected the DM and ADL content of the wheat straw. The increase of the ADL content in the autoclaved substrate is in agreement with previous reports (Tuyen *et al.*, 2012, 2013). The loss of water through evaporation during autoclaving increases the relative proportion of the cell wall components. The effect of the fungal treatment on the chemical composition of the wheat straw confirms the results of previous studies (Shrivastava *et al.*, 2011; Van Kuijk *et al.*, 2016a). The data presented here are again comparable to Tuyen *et al.* (2012) and small differences may be due to variation in the different batches of wheat straw. These data shows that by providing the same growth conditions and a comparable substrate composition, CS1 is capable of producing repeatable results. *C. subvermispora* has been regarded as a selective lignin degrader (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2016b) and to compare the selectivity between CS1 and CS2, the ratio of total cellulose and hemicellulose (carbohydrates) to lignin (C/L ratio) was calculated. A higher C/L ratio indicates more selective lignin degradation. After 7 weeks of colonization, CS1-treated straw showed a 1.2 times higher ( $P < 0.001$ ) C/L ratio, compared to the CS2-treated straw.

The changes in chemical composition can explain the IVGP profile of the fungal-treated straw, as well as the growth of the fungi. The correlations among all lignocellulose components with IVGP during the fungal colonization have been previously described (Tuyen *et al.*, 2012). In the present study, however, the IVGP was significantly ( $P < 0.001$ ) correlated to only hemicellulose ( $r = -0.85$ ) and lignin ( $r = -0.82$ ) content, but not to cellulose. Given both hemicellulose and cellulose may have positive effects on the IVGP, while lignin has a negative effect, a relationship between IVGP and C/L ratio was derived (Fig. 2.4). The IVGP of the CS 1 and CS2-treated straw was significantly correlated to their respective C/L ratios with the same Pearson's  $r$  values ( $r = 0.86$ ;  $P < 0.001$ ). A significant correlation between the IVGP and CP content was also observed ( $r = 0.59$ ;  $P < 0.001$ ). This observation suggests that the fungal treatment may have increased the availability and solubility of the protein for rumen microbes. However, this hypothesis needs to be tested with an incubation trial using N-free buffer medium (Cone *et al.*, 2009). Protein solubility and availability has a positive effect on the growth of the rumen microbes, hence the nutritive value of the substrate (Cone and Van Gelder, 1999). The ergosterol content was negatively correlated to the DM content of the substrate ( $r = -0.75$ ;

$P < 0.001$ ), but positively correlated to the C/L ratio ( $r = 0.73$ ;  $P < 0.001$ ) indicating that the fungal growth corresponds to the degradation of the DM and also the availability of the structural carbohydrates.

The presence of only laccase and MnP are consistent with the enzyme activities reported for *C. subvermispota* when they were grown in liquid media (Lobos *et al.*, 1994) or on biomass such as wood chips (Ferraz *et al.*, 2003; de Souza-Cruz *et al.*, 2004) and corn stover (Wan and Li, 2010). Nonetheless, there is limited information on the enzyme activities of *C. subvermispota*, particularly grown on wheat straw, for a direct comparison. In previous reports, there were large variations in the activities of these enzymes, which is not surprising as different substrates and/or fungal strains were used. A quantitative comparison is difficult as various assay methods are used and different units to express enzyme activities. Wan and Li (2010) also showed a peak activity of MnP and laccase during the first week of colonization of *C. subvermispota* on corn stover. In the present study, the laccase activity of CS1 is much lower compared to *C. subvermispota* grown on corn stover with an activity of 1500 U kg<sup>-1</sup> solids at week 1 (Wan and Li, 2010). Meanwhile, MnP of CS2 is also much lower when compared to *C. subvermispota* grown on corn stover (2200 U kg<sup>-1</sup> solids) (Wan and Li, 2010), or wood chips (156 U kg<sup>-1</sup> wood) (de Souza-Cruz *et al.*, 2004) at week 1.



**Fig. 2.4.** Relationship between cumulative *in vitro* gas production after 72 h with total carbohydrate to lignin (C/L) ratio for autoclaved wheat straw colonized by *Ceriporiopsis subvermispota* strains CS1 (O) and CS2 (▲) for up to 7 weeks. The continuous line is the regression line of CS1, and the dashed-line of CS2, with both being significant at  $P < 0.001$ .

According to Fernández-Fueyo *et al.* (2012), LiP activity might be expected in *C. subvermispora* as the genome of this fungus contains a LiP and a VP like gene, both likely representing an intermediate evolutionary state between LiP and VP. As LiP is considered important for an efficient ligninolysis, particularly related to its ability to cleave recalcitrant non-phenolic lignin, several mechanistic hypotheses have been proposed such as MnP-mediated lipid peroxidation (Jensen *et al.*, 1996) and involvement of laccase. The function of MnP was thought to be the least important for lignin degradation, as the chelated  $Mn^{3+}$  which is oxidized by MnP, can only act on phenolic lignin substructures. However, several authors provided evidences that MnP could promote the oxidation of non-phenolic lignin through lipid peroxidation (Bao *et al.*, 1994; Jensen *et al.*, 1996). Nishimura *et al.* (2012) identified the production of itaconic acids by *C. subvermispora* and showed that these compounds initiate lipid peroxidation with MnP. The latter might explain the substantial degradation of lignin by this fungus using mainly MnP enzymes. This process, however, is relatively slow and inefficient (Fernández-Fueyo *et al.*, 2012) compared to LiP. Laccase alone is incapable of depolymerizing macromolecular lignin due to its low redox potential (Hatakka and Hammel, 2010). However, in the presence of low molecular weight mediator compounds such as syringaldehyde and vanillin, the substrate range of laccase can be expanded which includes non-phenolic lignin (Christopher *et al.*, 2014). Therefore, laccase together with mediators compounds greatly improves delignification by the fungi (Hatakka and Hammel, 2010; Christopher *et al.*, 2014). Laccase has been shown as essential in lignin degradation for white-rot fungus *Pycnoporus cinnabarinus*, which lack both LiP and MnP. This information indicates that laccase may be more important in the degradation of lignin compared to MnP, when there is no LiP present.

At peak activity, the CS1-treated straw showed 2.5 times more laccase compared to CS2 while, on the other hand, CS2-treated straw showed 2 times more MnP. It can be assumed that CS1 may have higher lignin-degrading enzyme capabilities compared to CS2, which may explain the higher selectivity of this strain to degrade lignin. The ADL content showed no significant correlation to any lignin degrading enzyme. Thus, the correlation of these main enzymes with lignin remain unproven, which is in agreement with previous studies (Sharma and Arora, 2010; Shrivastava *et al.*, 2011). This outcome is due to the fact that, although these enzymes only have peak activities at week 1, the degradation of lignin continues throughout colonization. High activities of these main, lignin degrading enzymes may also indicate that the fungus start degrading lignin when all freely available carbohydrates become limiting at the beginning of colonization. This

will subsequently increase the accessibility of the lignocellulose complex to the action of other enzyme systems. It is worth noting that lignin degradation does not only involve peroxidases, such as LiP and MnP and phenol oxidases, such as laccase, but also other accessory enzymes and even non-enzymatic pathways (Dashtban *et al.*, 2010).

## CONCLUSION

In conclusion, the two strains of *C. subvermispota* were capable to selectively degrade lignin and improve *in vitro* degradability in rumen fluid of the wheat straw, with CS1 showing a higher potential than CS2. Both strains differ in their growth pattern and enzymatic activities as well as in the degree of selectivity in lignin degradation. For future investigations, assessing enzyme profiles, using more advanced techniques, such as secretome analysis, would provide better insight into the ability of a particular fungus. The observation that CS1 and CS2 clearly differ in the amount and timing of the production of enzymes involved in lignin degradation offers the opportunity to combine these activities in one strain by breeding. This new strain may have stronger growth characteristics and be more selective in degrading lignin, which can benefit the bioprocessing of lignocellulose biomass, not only for animal feed, but also other purposes such as biofuels production and biopulping.

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“

We can't solve problems by using the same kind of thinking we used when we created them.

”

- ALBERT EINSTEIN

# Screening of white-rot fungi for bioprocessing of wheat straw into ruminant feed

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## ABSTRACT

In this study, the biological variation for improvement of the nutritive value of wheat straw by 12 *Ceriporiopsis subvermispota*, 10 *Pleurotus eryngii* and 10 *Lentinula edodes* strains was assessed. Screening of the best performing strains within each species was made based on the *in vitro* degradability of fungal treated wheat straw. Wheat straw was inoculated with each strain for 7 weeks of solid state fermentation. Weekly samples were evaluated for *in vitro* gas production (IVGP) in buffered rumen fluid for 72 h. Out of the 32 fungal strains studied, 17 strains showed a significantly higher ( $P < 0.05$ ) IVGP compared to the control after 7 weeks (227.7 ml g<sup>-1</sup> OM). The three best *C. subvermispota* strains showed a mean IVGP of 297.0 ml g<sup>-1</sup> OM, while the three best *P. eryngii* and *L. edodes* strains showed a mean IVGP of 257.8 and 291.5 ml g<sup>-1</sup> OM, respectively. *C. subvermispota* strains show an overall high potential to improve the ruminal degradability of wheat straw, followed by *L. edodes* and *P. eryngii* strains. Large variation exists within and among different fungal species in the valorization of wheat straw, which offers opportunities to improve the fungal genotype by breeding.

**Keywords:** White-rot fungi; different strains/species; screening; *in vitro* gas production; degradability; wheat straw; ruminant

## INTRODUCTION

Exploring alternative feed ingredients for ruminant nutrition is important, not only for a sustainable animal production, but also to further ensure human food security. Agricultural biomass, such as wheat straw, is an attractive choice for this purpose. The use and challenges in the processing of this highly lignified biomass into a more digestible animal feed has been addressed in many studies (Shrivastava *et al.*, 2014; Van Kuijk *et al.*, 2015a). Different pretreatment methods have been used, including physical, chemical and biological methods (Sarnklong *et al.*, 2010; Isroi *et al.*, 2011). The goal of any pretreatment method is to break the lignin barrier and make cellulose and hemicellulose more accessible to fermentation in the rumen (Sarnklong *et al.*, 2010). Each method, however, has a varying effect on the composition and structure of cellulose, hemicellulose and lignin (Mosier *et al.*, 2005; Hendriks and Zeeman, 2009). Biological methods, including the use of fungi have attracted the interest of many researchers due to an increasing global demand for eco-friendly approaches (Dinis *et al.*, 2009; Sharma and Arora, 2014; Shrivastava *et al.*, 2011; Tuyen *et al.*, 2012).

Lignin has a negative impact on the degradation of fiber in ruminants (Moore and Jung, 2001). Degradation of lignin is a chemically difficult process since these polyphenolic compounds have a dense three-dimensional, highly branched structure that is linked with hemicellulose (Kubicek, 2013). White-rot fungi are the only organisms capable of degrading lignin aerobically to CO<sub>2</sub> and water (Hatakka and Hammel, 2010). This unique ability makes them favorable as candidates for the bioprocessing of highly lignified agricultural biomass into ruminant feed. However, not all fungal species exhibit a preferential degradation of lignin, i.e. selectively degrade lignin while utilizing low amounts of the structural carbohydrates. Tuyen *et al.* (2012) showed that out of 11 fungal species tested, only *Ceriporiopsis subvermispora* (Pilát) Gilb. & Ryvarden 1985, *Lentinula edodes* (Berkeley) Pegler 1975 and *Pleurotus eryngii* var. *eryngii* (DC.) QuéL 1872, showed a high lignin to cellulose loss ratio, which increased the *in vitro* degradability in rumen fluid of the treated wheat straw by 20 to 60% after 7 weeks of solid state incubation.

Bioprocessing of agricultural biomass, using white-rot fungi, is a complex process which is influenced by many factors, such as fungal species and strain, substrate and culture conditions (Van Kuijk *et al.*, 2015a). Several studies worked on the optimization of the process, such as exploring the best fungi-substrate combinations (Tuyen *et al.*, 2013), varying particle sizes of the substrate (Van Kuijk *et al.*, 2016a) and supplementing additives such as manganese and linoleic acid to the substrate (Van Kuijk *et al.*, 2016b). However, little work has been done on selecting the best fungal strain to maximize the

valorization of biomass for ruminant use. Fungi are diverse in their lignocellulose deconstruction mechanisms (Cragg *et al.*, 2015), which emphasize the need to not only investigate and screen for the best performing fungal species, but also different strains within a particular species. Most studies selected fungal strains based on their enzyme activity (Mata and Savoie, 1998; Cavallazzi *et al.*, 2004). However, correlating qualitative enzyme activities with the selectivity of a fungus was difficult (Sharma and Arora, 2010). Recently, we showed differences between two strains of *C. subvermispora* in improving the *in vitro* degradability of wheat straw, as well as in their growth and lignin-degrading enzyme characteristics (Nayan *et al.*, 2017). The ultimate goal of the ongoing research is to study the variation among fungal strains, which can be a valuable input for future breeding activities to expand the potential of fungi in the bioprocessing of agricultural biomass into ruminant feed. Therefore, the degradability of the substrate, as determined by the *in vitro* gas production (IVGP) (Cone *et al.*, 1996) was used as the main screening criterion.

The aim of the present study was to assess the biological variation among different strains within three species of white-rot fungi – *C. subvermispora*, *P. eryngii* and *L. edodes*, with respect to the formation of fungal biomass in time and the effect on IVGP. Three of the best strains for each species were selected based on IVGP and further compared for differences in the gas production kinetics and other fermentation characteristics; as well as changes in the mass balance of the wheat straw.

## MATERIALS AND METHODS

### Preparation of fungal strains and spawn

Different strains of 3 white-rot fungi species: *C. subvermispora* (CS; 12 strains), *P. eryngii* (PE; 10 strains) and *L. edodes* (LE; 10 strains) (Table 3.1) from the collection of the Plant Breeding Group, Wageningen University & Research, the Netherlands, were used. All strains were cultured on malt extract agar (malt extract 20 g l<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 0.5 g l<sup>-1</sup>, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g l<sup>-1</sup> and Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O 0.5 g l<sup>-1</sup> with a pH of 5.4) and incubated at 24°C until the mycelia colonized most of the agar surface. The spawn of each strain was prepared by placing a piece of the agar culture (1.5×2.0 cm) into a container with sterilized (20 min, 121°C) sorghum grains and incubated at 24°C for 5 weeks.



**Table 3.1.** List of strains from three different white-rot fungi species, with respective notations, used in the study.

Species/ Strain no	Original annotation	Origin
<b><i>Ceriporiopsis subvermispora</i></b>		
1*#	CBS 347.63	USA
2#	ACCC31512	China
3 <sup>a</sup>	AR-93	Forest Product Laboratory, USDA, USA
4	FP-104027-T	USA
5	FP-105752-Sp	USA
6	L-3292-R	USA
7	L-6133-Sp	USA
8	L-6332-Sp	USA
9	L-9186-Sp	USA
10	L-14807-Sp	USA
11	L-15225-Sp	USA
12	ME-485	USA
<b><i>Pleurotus eryngii</i></b>		
1	pl 03/04	Commercial cultivar
2*	pl 02/08	China
3	Mycelia2600	Commercial cultivar
4	P.eryngii 87	Unknown
5	AH03	Iran
6	AL04	Iran
7	BA19	Iran
8	F23	Iran
9	HE05	Iran
10	P02	Iran
<b><i>Lentinula edodes</i></b>		
1	sh 02/05	China
2	SP-1A	Germany
3	sh00/01	China
4	Somycel 4087	Commercial cultivar
5	ML 12	Japan
6	UGM	Indonesia
7* <sup>b</sup>	CCBAS389	Czech Republic
8	sh 03/08	Japan
9	msc03	Japan
10	LE (75)	Ghana

\* Reference strains as used by Tuyen *et al.* (2012).

# Homokaryon (no clamp connections).

<sup>a</sup> <https://www.fpl.fs.fed.us/research/centers/mycology/culture-collection.shtml>.

<sup>b</sup> <http://www.biomed.cas.cz/ccbas/fungi.htm>.

### Substrate preparation, fungi inoculation and sampling

A bale of organic wheat straw (*Triticum aestivum* L.) (300 kg; dry matter content 880.4 g kg<sup>-1</sup>) was purchased from a local farmer in the Netherlands and used for all incubations. Due to the limited capacity of the incubation chamber, treatments were conducted in three independent but adjacent periods with only one species (with different strains) for each period. Straw was chopped into approximately 3 cm pieces and soaked in water for 3 days at room temperature. After draining the excess of water for 5 h, the moisture content of each batch of straw was recorded. The wheat straw was then distributed into 185×185×78 mm micropropagation containers (Combiness, Nevele, Belgium), which is equipped with a depth-filtration strip on the lid, and the amount of wet straw in each container was adjusted to contain 90.2 ± 0.3 g of dry matter for all batches.

All containers were autoclaved at 121°C for 1 h and allowed to cool overnight at room temperature. Pre- and post-autoclaved wheat straw samples were taken for comparing the dry matter content. The handling and processing of the wheat straw was maintained in the same order throughout the experiment. The autoclaved straw was aseptically inoculated with the previously prepared spawn for each strain/species at 10% of the dry weight of the straw. Containers with inoculated straw and autoclaved straw (control) were incubated aerobically in triplicate at 24°C for 7 weeks in a climate-controlled chamber. Weekly samples were weighed, thoroughly mixed and ~5 g of fresh sample was taken for pH measurements. The remaining sample was freeze-dried and ground over a 1 mm sieve using a cross beater mill (100AN, Peppink, Olst, the Netherlands).

### Ergosterol estimation

Fungal biomass was estimated with an ergosterol assay (Niemenmaa *et al.*, 2008). In brief, 200 ± 10 mg sample was weighed into a glass tube and after extraction with 10% (1:9) KOH/methanol solution for 10 min, the tube was saponified at 80°C for 60 min. After cooling to room temperature, 1 ml distilled water and 2 ml hexane was added before each tube was thoroughly shaken. The hexane layer was subsequently collected and the extraction steps were repeated. The pooled hexane layer was dried in a vacuum evaporation system (Rapidvap, Kansas, MO, USA). The extracted ergosterol was re-dissolved in methanol and the solution was filtered into a high performance liquid chromatography (HPLC) vial for Waters HPLC-PDA analysis (Alliance HPLC system, Milford, USA). A reversed phase C18 column (250×4.6 mm, Phenomex aqua 5 µm) was used in the HPLC instrument and the liquid phase was 90% methanol and 10% (1:1) 2-

propanol/hexane. Cholecalciferol (vitamin D<sub>3</sub>) was used as an internal standard. The ergosterol peak was detected at 280 nm.

### ***In vitro* gas production**

All samples were subjected to *in vitro* gas production (IVGP), expressed as ml g<sup>-1</sup> organic matter (OM) (Cone *et al.*, 1996), where 0.5 ± 0.01 g of sample was incubated in 60 ml of buffered rumen fluid at 39 °C. The incubation was conducted in 250-ml bottles (Schott, Mainz, Germany) for 72 h and the gas production was registered automatically. At the end of the incubation, rumen fluid samples from each bottle (600 µl) were taken for the determination of volatile fatty acids (VFA) and ammonia-N (NH<sub>3</sub>-N). The remaining content in the bottles was filtered through a crucible and dried in an oven at 103°C to determine the dry matter (DM) content (ISO 6496, 1999) and the ash content in a furnace at 550°C for 3 h (ISO 5984, 2002). The organic matter (OM) digestibility was calculated as a percentage of digested OM from the pre-incubated sample.

Only samples of selected fungal strains/species (3 best performing strains from each species) were analyzed for VFA and NH<sub>3</sub>-N. Individual VFA concentrations were separated by gas chromatography using hydrogen as carrier gas, while reaction of ammonia with phenol and hypochlorite was measured spectrophotometrically at 623 nm for NH<sub>3</sub>-N determinations. The kinetic parameters were determined by fitting the gas production data to a biphasic model (Groot *et al.*, 1996), representing fermentation of the soluble (phase 1; 0 to 4 h) and the non-soluble fractions (phase 2; 4 to 48 h) according to Tuyen *et al.* (2012). Only parameters of phase 2 ( $A_2$ ,  $B_2$ ,  $C_2$ ,  $t_{Rm2}$ ,  $R_{m2}$ ) are presented here.  $A_2$  is the asymptotic gas production (ml g<sup>-1</sup> OM) of phase 2;  $B$  is the half time of the maximum gas production (h);  $C$  is a parameter to determine the steepness of the curve;  $t_{Rm}$  is the time of the maximum fractional rate of substrate degradation (h); and  $R_m$  the maximum fractional rate of substrate degradation (h<sup>-1</sup>).

### **Chemical analysis**

All freeze-dried samples were analyzed for DM and ash content. Only selected fungal strains/species samples were analyzed for nitrogen and fiber content. Nitrogen content was determined by the Kjeldahl method (ISO 5983, 2005) and crude protein was calculated as N × 6.25. Neutral detergent fiber (NDF) was determined using a heat-stable amylase (thermamylyl) and alcalase, using the standard procedures of Van Soest *et al.* (1991). Acid detergent fiber (ADF) and acid detergent lignin (ADL) were also determined using the standard method (Van Soest *et al.*, 1991). Hemicellulose was calculated as the

difference between NDF and ADF, while cellulose was calculated as the difference between ADF and ADL. Absolute amounts (g) of each component was calculated from the remaining amount (g) of freeze-dried sample which was corrected for the dry matter content.

### Statistics

Data were analyzed per species using the general linear model in SAS 9.3, followed by a post-hoc multiple comparison using least significance differences. The statistical model was as follows:

$$Y_{ij} = \mu + ST_i + \tau_j + (ST \times \tau)_{ij} + \varepsilon_{ij}$$

where  $Y_{ij}$  = response variable,  $\mu$  = overall mean,  $ST_i$  = the effect of strain  $i$ ,  $\tau_j$  = effect of week  $j$ ,  $(ST \times \tau)_{ij}$  = interaction of strain  $i$  and week  $j$ , and  $\varepsilon_{ij}$  = residual error with a mean of 0 and variance  $\sigma^2$ . For assessment and comparison of selected strains across species, nested analysis of variance without time interaction was conducted using the following statistical model:

$$Y_{ijk} = \mu + SP_i + ST_{j(i)} + \tau_{k(i,j)} + \varepsilon_{ijk}$$

where  $Y_{ijk}$  = response variable  $ijk$ ,  $\mu$  = overall mean,  $SP_i$  = the effect of species  $i$ ,  $ST_{j(i)}$  = the effect of strain  $j$  nested within species  $i$ ,  $\tau_{k(i,j)}$  = effect of week  $k$ , and  $\varepsilon_{ijk}$  = residual error with a mean of 0 and variance  $\sigma^2$ .  $SP_i$  was considered a fixed effect,  $ST_{j(i)}$  and  $\tau_{k(i,j)}$  as random effects. The CONTRAST statement was also used in SAS to allow comparison among different species group. Pearson Product-Moment Correlation ( $r$ ) coefficients were determined among the measured variables. Probability values below 5% were considered significant. The ergosterol data were fitted to a polynomial regression models. A linear model was fitted first to the ergosterol data of a strain where after a quadratic term was added. If the quadratic term was significant, a cubic term was added to the model. The model with the highest significant ( $P < 0.05$ ) coefficient was taken as the best fit model. The rate of ergosterol changes over time ( $\delta_{\text{Erg}}$ ) was calculated by substituting week 7 ( $x_2 = 7$ ) and week 0 ( $x_1 = 0$ ) into respective polynomial functions,  $f(x)$ , and dividing the value by 7.

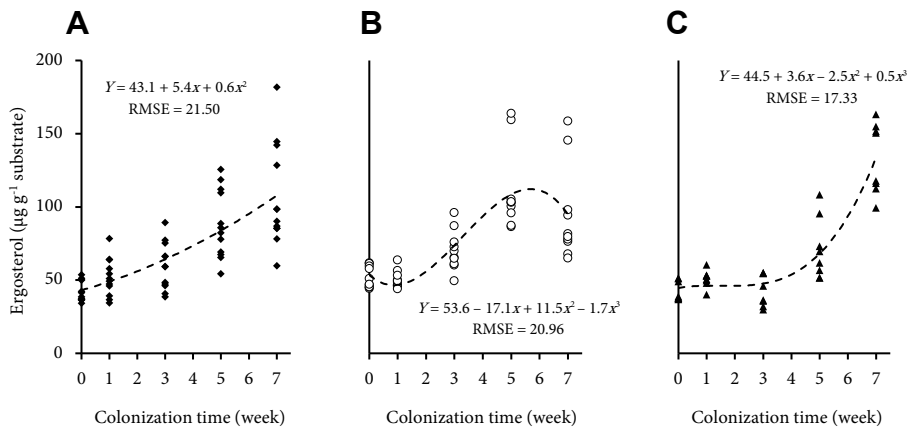
## RESULTS

### Fungal growth and changes in the pH of substrate

Fig. 3.1 shows the pattern of change in ergosterol content in time for all fungal strains within each species. In general, the ergosterol concentrations in fungal treated straw

showed a curvilinear pattern for all strains, with a lag phase up to 3 weeks for *L. edodes*. The ergosterol data of each strain were fitted to a polynomial regression model to describe the growth pattern and to estimate the changes in ergosterol ( $\delta_{\text{Erg}}$ ) over time (Table 3.2). Most of *C. subvermispota* strains showed quadratic and linear growth patterns. All *P. eryngii* and *L. edodes* strains (except LE1 and LE9) mostly followed a cubic function. Four *C. subvermispota* strains (CS3, CS7, CS4 and CS11), showed a significantly higher ( $P < 0.001$ )  $\delta_{\text{Erg}}$  compared to other CS strains and CS1 had the lowest  $\delta_{\text{Erg}}$ . Among *P. eryngii* strains, PE5 had the highest  $\delta_{\text{Erg}}$ . High  $\delta_{\text{Erg}}$  values among *L. edodes* strains were observed for LE10, LE1, LE2 and LE3. Two *L. edodes* strains (LE4 and LE5), showed irregular changes with the ergosterol contents of 271.4 and 449.4  $\mu\text{g g}^{-1}$ , respectively, despite no apparent mycelium being observed after 7 weeks. These data were excluded from the analysis.

Typical changes in pH for all fungal strains/species are shown in Fig. 3.2. *C. subvermispota* strains significantly ( $P < 0.001$ ) decreased the pH of wheat straw from 5.00 to 3.52, while *L. edodes* strains (excluding LE4 and LE5) decreased ( $P < 0.001$ ) the pH to 3.95. For *P. eryngii* strains, there were significant ( $P < 0.001$ ) increases in pH up to week 3, before it significantly decreased to pH 4.46 at week 7. After 7 weeks of colonization, the pH of wheat straw treated with LE4 and LE5 increased to pH 6.87 and 7.17, respectively.



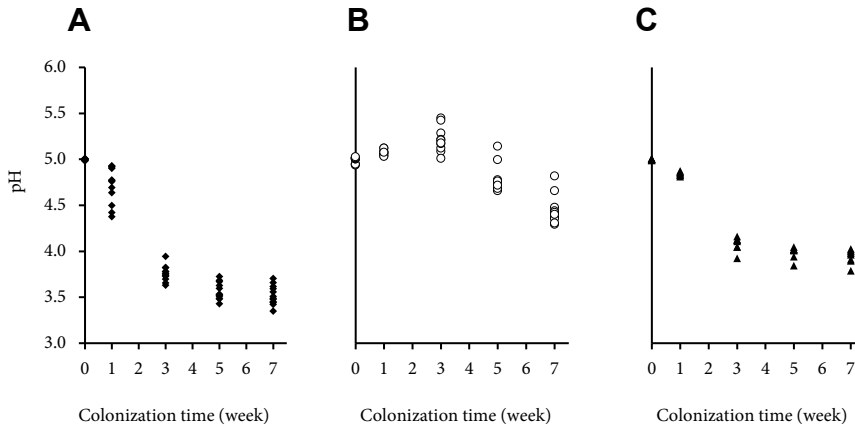
**Fig. 3.1.** Growth patterns of *Ceriporiopsis subvermispota* (A), *Pleurotus eryngii* (B) and *Lentinula edodes* (C) strains, as estimated by the ergosterol amount during 7 weeks of colonization on wheat straw. LE4 and LE5 were omitted from the graph, due to irregular changes. Dashed-lines indicate the average polynomial trend for each species with respective model and root mean square error (RMSE) are shown. The Y-axes are equally scaled.

**Table 3.2.** Changes in ergosterol ( $\delta_{\text{Erg}}$ ) contents of different fungal strains grown on wheat straw for 7 weeks and their respective regression coefficients.

Species/ strains	Erg <sub>week 7</sub> ( $\mu\text{g g}^{-1}$ )	$\delta_{\text{Erg}}$	Regression coefficients <sup>a</sup>				$R^2$	RMSE
			$\beta_0$	$\beta_1$	$\beta_2$	$\beta_3$		
<b><i>Ceriporiopsis subvermispora</i></b>								
CS1	59.6 <sup>a</sup>	1.6 <sup>f</sup>	49.1	-1.6	0.5		0.72	3.21
CS2	98.4 <sup>c</sup>	7.2 <sup>d</sup>	44.1	7.2			0.67	13.97
CS3	181.6 <sup>f</sup>	18.4 <sup>a</sup>	51.9	-1.8	2.9		0.98	7.73
CS4	144.6 <sup>e</sup>	13.0 <sup>bc</sup>	53.2	13.0			0.83	16.24
CS5	85.2 <sup>b</sup>	7.4 <sup>d</sup>	34.9	-0.4	1.1		0.94	5.56
CS6	78.0 <sup>b</sup>	4.6 <sup>e</sup>	44.0	4.6			0.60	10.37
CS7	142.1 <sup>e</sup>	14.6 <sup>b</sup>	41.9	6.6	1.2		0.97	7.14
CS8	98.3 <sup>c</sup>	7.8 <sup>d</sup>	45.1	-23.9	10.8	-0.9	0.93	8.03
CS9	85.3 <sup>b</sup>	7.2 <sup>d</sup>	38.0	7.2			0.90	6.72
CS10	86.8 <sup>b</sup>	7.4 <sup>d</sup>	34.2	7.4			0.94	5.29
CS11	128.4 <sup>d</sup>	12.7 <sup>c</sup>	40.2	21.4	-1.2		0.97	6.74
CS12	90.2 <sup>bc</sup>	7.3 <sup>d</sup>	38.4	12.6	-0.8		0.91	6.63
<b><i>Pleurotus eryngii</i></b>								
PE1	67.9 <sup>ab</sup>	2.4 <sup>e</sup>	63.4	-37.1	17.7	-1.8	0.87	8.43
PE2	81.5 <sup>cd</sup>	2.4 <sup>e</sup>	64.6	-38.9	16.8	-1.6	0.91	7.07
PE3	76.4 <sup>abc</sup>	2.3 <sup>e</sup>	60.3	-28.1	13.6	-1.3	0.87	7.68
PE4	145.3 <sup>f</sup>	8.8 <sup>b</sup>	60.6	-16.3	14.5	-1.5	0.89	16.52
PE5	158.6 <sup>f</sup>	15.2 <sup>a</sup>	52.9	-28.6	19.5	-1.9	0.95	14.19
PE6	64.9 <sup>a</sup>	2.5 <sup>e</sup>	48.7	-0.6	4.2	-0.5	0.84	6.37
PE7	77.9 <sup>bc</sup>	4.8 <sup>d</sup>	44.4	1.8	3.6	-0.5	0.93	5.22
PE8	79.6 <sup>bc</sup>	4.6 <sup>d</sup>	47.9	-14.2	10.7	-1.1	0.92	7.57
PE9	94.2 <sup>de</sup>	6.9 <sup>c</sup>	46.8	-2.0	5.9	-0.7	0.96	5.28
PE10	98.0 <sup>e</sup>	7.4 <sup>c</sup>	46.0	-7.2	8.0	-0.9	0.98	3.73
<b><i>Lentinula edodes</i></b>								
LE1	163.1 <sup>a</sup>	16.0 <sup>a</sup>	51.7	-12.0	4.0		0.95	11.32
LE2	151.5 <sup>a</sup>	14.4 <sup>ab</sup>	50.8	6.4	-4.1	0.8	0.95	10.75
LE3	150.3 <sup>a</sup>	14.0 <sup>ab</sup>	51.8	-24.0	10.9	-0.8	0.97	8.19
LE6	99.2 <sup>b</sup>	8.5 <sup>c</sup>	40.2	9.3	-5.1	0.7	0.94	6.75
LE7	116.0 <sup>c</sup>	11.0 <sup>bc</sup>	40.2	3.0	-3.3	0.6	0.95	8.37
LE8	112.3 <sup>c</sup>	10.2 <sup>c</sup>	41.2	12.1	-6.9	0.9	0.92	9.43
LE9	117.6 <sup>c</sup>	9.8 <sup>c</sup>	47.0	-12.7	3.2		0.85	13.55
LE10	154.9 <sup>a</sup>	16.8 <sup>a</sup>	38.6	29.0	-11.8	1.4	0.92	14.50

Values with different superscripts within column of the same species are significantly ( $P < 0.05$ ) different. Erg<sub>week 7</sub>: Ergosterol amounts after 7 weeks of colonization;  $\delta_{\text{Erg}}$ : Calculated rate of ergosterol changes ( $\mu\text{g g}^{-1}$  per week) over 7 weeks using respective regression functions. LE4 and LE5 are omitted from the table due to irregular changes.

<sup>a</sup> Regression coefficients of the polynomial functions with intercept  $\beta_0$  with respective coefficient of determination ( $R^2$ ) and root mean square error (RMSE).

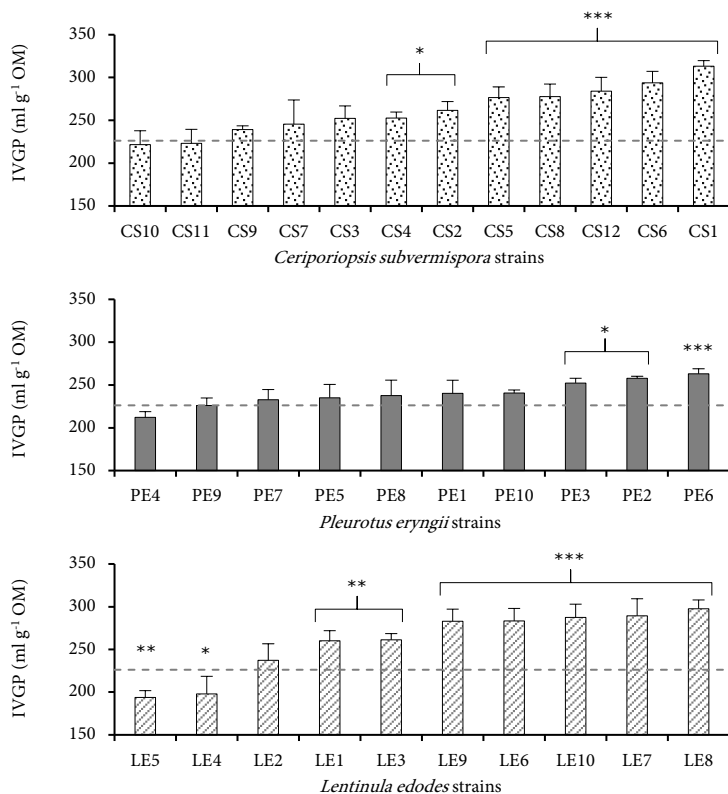


**Fig. 3.2.** Trends in pH changes of wheat straw treated with different strains of *Ceriporiopsis subvermispora* (A), *Pleurotus eryngii* (B) and *Lentinula edodes* (C) during 7 weeks of colonization. LE4 and LE5 were omitted from the graph due to irregular changes. The Y-axes are equally scaled.

### *In vitro* gas production

*In vitro* gas production (IVGP) was used to select the best performing fungal strains for each species. Overall, 17 out of 32 fungal strains tested, significantly ( $P < 0.05$ ) improved the IVGP of wheat straw after 7 weeks of colonization (Fig. 3.3). Seven strains of *C. subvermispora* significantly ( $P < 0.05$ ) increased the IVGP of the wheat straw, with CS1, CS6, CS12, CS8 and CS5 being highly significant ( $P < 0.001$ ) compared to untreated straw. Fermentation of CS1-treated wheat straw produced most gas (313.2 ml g<sup>-1</sup> OM), followed by CS6 and CS12 (293.6 and 284.2 ml g<sup>-1</sup> OM, respectively). Among *P. eryngii* strains, 3 strains – PE6, PE2 and PE3 significantly ( $P < 0.05$ ) increased the IVGP of the wheat straw. PE6 had the highest IVGP (263.0 ml g<sup>-1</sup> OM), followed by PE2 and PE3 with a mean IVGP of 257.9 and 252.4 ml g<sup>-1</sup> OM, respectively. Seven strains of *L. edodes* significantly ( $P < 0.05$ ) increased the IVGP of the straw where five of them were highly significant ( $P < 0.001$ ). LE8 showed the highest IVGP (297.7 ml g<sup>-1</sup> OM), followed by LE7 (289.4 ml g<sup>-1</sup> OM) and LE10 (287.3 ml g<sup>-1</sup> OM). LE4 and LE5, for which irregular changes in growth and pH were observed, showed a significantly ( $P < 0.05$ ) lower IVGP compared to the untreated straw.

Three strains from each species with the highest IVGP values were selected for further assessment and comparison across species. The selected strains (in order of IVGP values) were CS1, CS6 and CS12 for *C. subvermispora*; PE6, PE2 and PE3 for *P. eryngii*; and LE8, LE7 and LE10 for *L. edodes*.



**Fig. 3.3.** Total *in vitro* gas production (IVGP) of wheat straw treated with different fungal strains (within species) after 7 weeks of colonization. The IVGP values were sorted ascendingly within each species. Dashed-line marks the gas production of the control (untreated straw) and error bars indicate the standard deviation. Asterisks show significant differences from the control at  $P < 0.001$  (\*\*\*),  $P < 0.01$  (\*\*) and  $P < 0.05$  (\*).

### Gas production kinetics and fermentation characteristics

The kinetic parameters of the second (non-soluble) phase of the gas production curves (4 to 48 h) for the selected fungal strains are summarized in Table 3.3. Compared to the untreated wheat straw, all fungal-treated wheat straw showed significantly ( $P < 0.01$ ) higher  $A_2$  values (except PE2 and PE3) and significantly ( $P < 0.001$ ) lower  $B_2$  values, indicating a higher and faster fermentation of the substrate. High  $A_2$  and low  $B_2$  were accompanied by significantly ( $P < 0.01$ ) lower  $t_{fm2}$  and higher  $R_{m2}$  values for all fungal-treated straw samples, compared to the control. Among different species, *C.*



**Table 3.3.** The effects of wheat straw treated with different fungal strains/species on the *in vitro* organic matter (IVOM) digestibility, kinetic parameters of phase 2 of the gas production, volatile fatty acids (VFA) and ammonia production after 7 weeks.

Species (Sp)/ Strains (St)	IVOM dig (%)	Kinetic parameters						VFA (mmol g <sup>-1</sup> OM)			HAC/ HPr	NH <sub>3</sub> -N (mg g <sup>-1</sup> OM)
		A <sub>2</sub>	B <sub>2</sub>	C <sub>2</sub>	t <sub>90%</sub>	R <sub>90%</sub>	HAC	HPr	HBu	TVFA		
Control	61.5 <sup>a</sup>	188.9 <sup>a</sup>	17.47 <sup>f</sup>	2.45 <sup>cde</sup>	20.34 <sup>e</sup>	0.071 <sup>a</sup>	8.59 <sup>cd</sup>	2.72 <sup>a</sup>	1.39 <sup>ef</sup>	13.42 <sup>c</sup>	3.16 <sup>g</sup>	54.94 <sup>d</sup>
<b><i>Ceriporiopsis subvermispora</i></b>												
CS1	82.1 <sup>b</sup>	242.4 <sup>de</sup>	13.51 <sup>b</sup>	2.53 <sup>c</sup>	15.97 <sup>ab</sup>	0.096 <sup>f</sup>	8.57 <sup>cd</sup>	2.84 <sup>b</sup>	1.22 <sup>c</sup>	13.32 <sup>c</sup>	3.02 <sup>e</sup>	44.94 <sup>b</sup>
CS6	83.3 <sup>b</sup>	237.7 <sup>de</sup>	12.40 <sup>a</sup>	2.60 <sup>ef</sup>	14.85 <sup>a</sup>	0.108 <sup>g</sup>	8.40 <sup>cd</sup>	2.71 <sup>a</sup>	1.20 <sup>c</sup>	13.00 <sup>ab</sup>	3.10 <sup>f</sup>	45.69 <sup>bc</sup>
CS12	76.5 <sup>bc</sup>	246.4 <sup>e</sup>	15.47 <sup>de</sup>	2.68 <sup>f</sup>	18.75 <sup>d</sup>	0.089 <sup>e</sup>	9.19 <sup>e</sup>	3.05 <sup>c</sup>	1.40 <sup>f</sup>	14.41 <sup>d</sup>	3.01 <sup>e</sup>	54.29 <sup>d</sup>
<b><i>Pleurotus eryngii</i></b>												
PE6	67.8 <sup>a</sup>	211.3 <sup>bc</sup>	14.49 <sup>e</sup>	2.21 <sup>a</sup>	15.76 <sup>ab</sup>	0.077 <sup>bc</sup>	8.59 <sup>cd</sup>	3.07 <sup>c</sup>	1.33 <sup>d</sup>	13.63 <sup>c</sup>	2.80 <sup>e</sup>	50.64 <sup>cd</sup>
PE2	66.7 <sup>a</sup>	197.4 <sup>ab</sup>	15.18 <sup>cd</sup>	2.30 <sup>b</sup>	17.04 <sup>bc</sup>	0.077 <sup>bc</sup>	8.28 <sup>bc</sup>	2.86 <sup>b</sup>	1.35 <sup>de</sup>	13.15 <sup>bc</sup>	2.90 <sup>d</sup>	53.33 <sup>d</sup>
PE3	67.8 <sup>a</sup>	191.3 <sup>a</sup>	15.16 <sup>cd</sup>	2.31 <sup>b</sup>	17.04 <sup>bc</sup>	0.077 <sup>bc</sup>	8.34 <sup>bc</sup>	2.88 <sup>b</sup>	1.37 <sup>ef</sup>	13.25 <sup>bc</sup>	2.90 <sup>d</sup>	53.82 <sup>d</sup>
<b><i>Lentinula edodes</i></b>												
LE8	75.6 <sup>bc</sup>	228.2 <sup>d</sup>	15.20 <sup>cd</sup>	2.40 <sup>cd</sup>	17.48 <sup>c</sup>	0.080 <sup>d</sup>	8.18 <sup>abc</sup>	3.25 <sup>d</sup>	1.14 <sup>ab</sup>	13.17 <sup>bc</sup>	2.51 <sup>a</sup>	39.54 <sup>a</sup>
LE7	75.6 <sup>bc</sup>	225.1 <sup>cd</sup>	14.54 <sup>c</sup>	2.37 <sup>bc</sup>	16.61 <sup>bc</sup>	0.083 <sup>d</sup>	8.13 <sup>ab</sup>	3.25 <sup>d</sup>	1.15 <sup>b</sup>	13.12 <sup>abc</sup>	2.50 <sup>a</sup>	38.87 <sup>a</sup>
LE10	72.8 <sup>ac</sup>	224.1 <sup>cd</sup>	16.05 <sup>e</sup>	2.47 <sup>de</sup>	18.75 <sup>d</sup>	0.078 <sup>cd</sup>	7.99 <sup>a</sup>	3.10 <sup>c</sup>	1.11 <sup>a</sup>	12.78 <sup>a</sup>	2.58 <sup>b</sup>	40.10 <sup>ab</sup>
RMSE	4.36	10.11	0.45	0.05	0.69	0.002	0.14	0.04	0.02	0.19	0.03	3.15
<b><i>P</i> values</b>												
Sp	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
St (Sp)	<0.001	0.034	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Time (St Sp)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.223

Values with different superscripts within column are significantly ( $P < 0.05$ ) different. A<sub>2</sub>: asymptotic gas production (ml g<sup>-1</sup> OM) at phase 2; B<sub>2</sub>: half time of the maximum gas production (h); C<sub>2</sub>: parameters determine curvature of the graph; t<sub>90%</sub>: time of the maximum fractional rate of substrate degradation (h); R<sub>90%</sub>: maximum fractional rate of substrate degradation (h<sup>-1</sup>); HAC: acetic acid; HPr: propionic acid; HBu: butyric acid; NH<sub>3</sub>-N: ammonia-N; RMSE: root mean square error; TVFA: total volatile fatty acids including iso-acids.

*subvermispora* strains showed a significant contrast ( $P < 0.001$ ) to the *P. eryngii* strains in all kinetic parameters. Other contrast wise comparisons among different fungal species showed significant differences ( $P < 0.05$ ), except for  $A_2$  values for *C. subvermispora* vs. *L. edodes* strains, and  $R_{m2}$  for *P. eryngii* vs. *L. edodes* strains. The increases in IVGP correlated with the increases in *in vitro* OM digestibility ( $r = 0.81$ ;  $P < 0.001$ ).

All fungal-treated wheat straw showed a decrease in acetate (except for CS12) during fermentation in rumen fluid, but an increase in propionate production (Table 3.3). This observation was also reflected by the changes in acetate/propionate ratio with the lowest values observed for *L. edodes* strains, followed by *P. eryngii* and *C. subvermispora* strains. All species were significantly ( $P < 0.001$ ) different to each other in their acetate/propionate ratio. There was a significant ( $P < 0.001$ ) decrease in  $\text{NH}_3\text{-N}$  production for *C. subvermispora* (except CS12) and *L. edodes* strains. The changes observed in all *P. eryngii* strains, however, were not significant. *L. edodes* strains showed the lowest  $\text{NH}_3\text{-N}$ , followed by *C. subvermispora* and *P. eryngii* strains. Contrast wise, strains were significantly ( $P < 0.001$ ) different from each other.

### Mass balance

The control, untreated wheat straw contained  $213.8 \text{ g kg}^{-1}$  of DM and  $33.3 \text{ g kg}^{-1}$  ash (on DM basis) with an absolute OM amount of  $87.2 \pm 0.3 \text{ g}$ . All mass balance data were presented as g per 100 g of this starting OM. The mass balance data of the selected fungal species/strains are summarized in Table 3.4. All fungal pretreatments decreased the amount of OM of the wheat straw by 1.6 to 7.8%, with the decreases in CS6 and CS12-treated straw were not significant. The fungal pretreatment resulted in 6.3 to 30.1% increase in the amount of crude protein. All fungi significantly ( $P < 0.001$ ) degraded ADL and hemicellulose in the wheat straw. The largest decrease in the amount of ADL and hemicellulose was observed in CS1-treated straw with 52.2 and 49.7% lower ADL and hemicellulose, respectively, compared to the control. The absolute amount of cellulose, however, did not change significantly, except for CS1. Although CS1-treated straw showed a decrease in cellulose, it contained the highest “enrichment” of total carbohydrates, i.e. highest carbohydrate to lignin ratio (C/L) in the remaining material. Wheat straw treated with *C. subvermispora* strains (except CS12) showed an overall high C/L, followed by *L. edodes* and *P. eryngii* strains. The total mass which was not identified ranged from 7.8 to 24.4% from the total amount of OM.

**Table 3.4.** The effects of wheat straw treated with different fungal strains/species on the amount of nutrients (g/ 100 g of starting organic matter) after 7 weeks of colonization.

Strains (St) / Species (Sp)	Amount in remains (g/ 100 g of starting OM)					C/L in remains			Percentage degraded (%)			
	OM	Cell	Hcell	ADL	CP	N.I.	Cell	Hcell	ADL	Cell	Hcell	ADL
Control	100.0 <sup>d</sup>	51.7 <sup>bc</sup>	30.1 <sup>f</sup>	8.8 <sup>g</sup>	1.7 <sup>a</sup>	7.8 <sup>a</sup>	9.35 <sup>a</sup>					
<b><i>Cetiporiopsis subvermispora</i></b>												
CS1	92.7 <sup>a</sup>	48.6 <sup>a</sup>	15.1 <sup>a</sup>	4.2 <sup>a</sup>	2.2 <sup>bc</sup>	22.7 <sup>g</sup>	15.26 <sup>c</sup>	6.0 <sup>d</sup>	49.7 <sup>c</sup>	52.2 <sup>f</sup>		
CS6	98.3 <sup>cd</sup>	52.4 <sup>cd</sup>	17.0 <sup>b</sup>	4.5 <sup>a</sup>	1.8 <sup>ab</sup>	22.6 <sup>g</sup>	15.38 <sup>c</sup>	-1.5 <sup>ab</sup>	43.6 <sup>d</sup>	48.4 <sup>f</sup>		
CS12	98.4 <sup>cd</sup>	50.5 <sup>b</sup>	20.5 <sup>c</sup>	7.2 <sup>f</sup>	2.1 <sup>abc</sup>	18.1 <sup>f</sup>	9.86 <sup>a</sup>	2.4 <sup>c</sup>	31.8 <sup>c</sup>	17.5 <sup>a</sup>		
<b><i>Pleurotus eryngii</i></b>												
PE6	97.0 <sup>bc</sup>	52.9 <sup>d</sup>	24.1 <sup>c</sup>	6.6 <sup>c</sup>	2.1 <sup>abc</sup>	11.3 <sup>b</sup>	11.68 <sup>bc</sup>	-2.3 <sup>a</sup>	20.0 <sup>a</sup>	24.6 <sup>b</sup>		
PE2	95.7 <sup>b</sup>	51.0 <sup>bc</sup>	22.3 <sup>d</sup>	6.5 <sup>c</sup>	2.2 <sup>bc</sup>	13.7 <sup>c</sup>	11.27 <sup>b</sup>	1.2 <sup>bc</sup>	26.1 <sup>b</sup>	25.7 <sup>b</sup>		
PE3	95.9 <sup>bc</sup>	50.8 <sup>b</sup>	23.3 <sup>de</sup>	6.4 <sup>de</sup>	2.2 <sup>c</sup>	13.3 <sup>c</sup>	11.61 <sup>bc</sup>	1.7 <sup>c</sup>	22.7 <sup>ab</sup>	27.1 <sup>bc</sup>		
<b><i>Lentinula edodes</i></b>												
LE8	92.2 <sup>a</sup>	51.4 <sup>bcd</sup>	17.5 <sup>b</sup>	5.2 <sup>b</sup>	2.1 <sup>abc</sup>	16.0 <sup>de</sup>	13.17 <sup>d</sup>	0.5 <sup>abc</sup>	42.1 <sup>d</sup>	40.1 <sup>c</sup>		
LE7	92.6 <sup>a</sup>	51.3 <sup>bc</sup>	17.2 <sup>b</sup>	5.5 <sup>bc</sup>	2.1 <sup>abc</sup>	16.6 <sup>ef</sup>	12.45 <sup>cd</sup>	0.7 <sup>bc</sup>	43.0 <sup>d</sup>	37.1 <sup>de</sup>		
LE10	93.1 <sup>a</sup>	51.1 <sup>bc</sup>	19.6 <sup>c</sup>	5.9 <sup>cd</sup>	2.2 <sup>bc</sup>	14.4 <sup>cd</sup>	12.02 <sup>bc</sup>	1.2 <sup>bc</sup>	34.8 <sup>c</sup>	32.8 <sup>cd</sup>		
RMSE	1.42	0.90	0.79	0.31	0.24	0.97	0.52	1.76	2.60	3.60		
<i>P</i> values												
Sp	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
St (Sp)	<0.001	<0.001	<0.001	<0.001	0.096	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Time (St Sp)	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values with different superscripts within column are significantly ( $P < 0.05$ ) different. ADL: acid detergent lignin; Cell: cellulose; C/L: total carbohydrate (cellulose + hemicellulose) to lignin ratio; CP: crude protein; Hcell: hemicellulose; N.I.: amount of non-identified OM (not measured); OM: organic matter; RMSE: root mean square error.

## DISCUSSION

A complete colonization of the substrate is a prerequisite for an effective fungal pretreatment (Van Kuijk *et al.*, 2015a). The ergosterol content of the treated wheat straw in the present study varied greatly among the different fungal strains/species, which has also been reported in other studies (Klamer and Bååth, 2004; Niemenmaa *et al.*, 2008). A simple polynomial regression was used as an empirical approach to compare the growth rate of different strains within species. Other mathematical approaches have also been used (Mirón *et al.*, 2002; Gutarowska and Zakowska, 2009), however a longer lag phase in our study contradicts the assumption made for these liquid-culture based models that growth should mainly occur at the beginning of the colonization. Fungi undergo a lag phase as a physiological preparation for an exponential phase, which depends on inoculum and available nutrients (Griffin, 1996). A longer lag phase was observed for CS1 and CS2, compared to a previous study which used the same strains grown under similar conditions (Nayan *et al.*, 2017). This was expected as the present study used 2.6 times more substrate (fresh weight basis) compared to the latter study.

The present study shows that *L. edodes* strains had a longer lag time compared to *C. subvermispora* and *P. eryngii* strains. For *P. eryngii* strains, there was an indication of a decrease in fungal biomass after week 5. Since ergosterol is relatively stable in substrate (Mille-Lindblom *et al.*, 2004), this observation indicates that the fungi recycled mycelium, probably due to a decline in their ability to obtain nutrients from the substrate. A continuing decrease in the pH of the substrate (Fig. 3.2) signals the generation of metabolites that lower the pH, indicating a persistent formation of new biomass at the expense of old biomass (Falconer *et al.*, 2005). Meanwhile, it is difficult to provide an explanation for the irregular changes observed in the ergosterol contents of LE4 and LE5 treated straw. A visual observation of these samples provided no indication for the presence of green molds (a sign of contamination). An increase in pH to near neutral and slightly alkaline for LE4 and LE5, respectively, might indicate that these fungi did not grow well on wheat straw or a probable bacterial contamination due to poor fungal growth. A decrease in substrate pH during the fungal colonization is a characteristic of lignin-degrading enzyme secretions (Liers *et al.*, 2011), in addition to the production of organic acids (Hofrichter *et al.*, 1999).

Degradability of treated wheat straw in rumen fluid, as measured using the IVGP technique (Cone *et al.*, 1996), was used here (Fig. 3.3) as a screening criterion for the best performing fungal strains. This accurate time-related technique has been widely used in feed evaluation research, including in assessing the degradability of fungal-treated

agricultural biomass (Tuyen *et al.*, 2013; Van Kuijk *et al.*, 2015a). Other important parameters have also been used as screening criteria such as selective delignification which resulted in a high C/L, growth characteristics and enzyme activities (Li *et al.*, 2008; Chang *et al.*, 2012; Cruz Ramírez *et al.*, 2012). Our choice of using the IVGP for strains screening is based on the fact that it provides a good indication for the improvement in the nutritive value of wheat straw in a convenient and robust manner. Moreover, an increase in IVGP has been correlated with an increase of C/L in the substrate (Karunanandaa and Varga, 1996a; Tuyen *et al.*, 2012). A selective fungus is desirable for bioprocessing of wheat straw into ruminant feed.

CS1, PE2 and LE7 were the same fungal strains as used in previous studies (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015b; Nayan *et al.*, 2017). The IVGP of CS1-treated wheat straw in the present study (313.2 ml g<sup>-1</sup> OM) was slightly higher than previously reported (300 to 305 ml g<sup>-1</sup> OM). The IVGP of PE2-treated straw (257.9 ml g<sup>-1</sup> OM) was also within the range of previously reported for wheat straw treated with this strain (250 to 260 ml g<sup>-1</sup> OM). The IVGP of LE7-treated wheat straw reported here (289.4 ml g<sup>-1</sup> OM) was slightly higher than that of Tuyen *et al.* (2012) (270 to 280 ml g<sup>-1</sup> OM). Van Kuijk *et al.* (2015b) reported an IVGP value of 311.3 ml g<sup>-1</sup> OM when wheat straw was treated with this strain for 12 weeks. All selected IVGP data were subjected to a biphasic model (Groot *et al.*, 1996) to assess the kinetic parameters of the gas production curves. This curve-fitting tool has been shown to be useful in comparing the gas production profiles of wheat straw treated with two strains of *C. subvermispota*, although the total IVGP values of both fungi were not significantly different (Nayan *et al.*, 2017). We present only parameters of the second phase as it is related to the fermentation of structural carbohydrates (Groot *et al.*, 1996; Cone *et al.*, 1997). Results show that wheat straw treated with *C. subvermispota* strains had an overall better gas production profile – higher  $A_2$  and lower  $B_2$ , followed by *L. edodes* and *P. eryngii* strains.

Fermentation of a low quality, highly fibrous feed results in an increase in acetate production, while an increase in rapidly fermentable carbohydrates will result in an increase in propionate production (Van Houtert, 1993). Other factors that can also affect the acetate/propionate ratio, including substrate composition and availability, as well as microbial species present (Dijkstra, 1994). An increase in IVGP indicates that the fungal pretreatment modifies the complexity of the cell wall and transforms the wheat straw into a more fermentable fiber source. The effect of a change from complex to easily digestible fiber sources (by means of chemical pretreatment) on the VFA production has been demonstrated by Griffith *et al.* (2016). They reported a similar decrease in acetate and

acetate/propionate ratio, as well as an increase in propionate molar proportion. Similar results were also reported for rice straw treated with *Cyathus stercoreus* (Karunanandaa and Varga, 1996b). However, Karunanandaa and Varga (1996a) reported an increased acetate and acetate/propionate ratio in rice straw treated with *Phanerochaete chrysosporium*. A likely explanation is that this fungus is considered to be less selective and reduces the amount of carbohydrates considerably (Tuyen *et al.*, 2012). A lower NH<sub>3</sub>-N was also reported for fungal treated rice straw, compared to a control (Karunanandaa and Varga, 1996b). During the *in vitro* incubation, a decrease in NH<sub>3</sub>-N concentration in the rumen fluid was followed by a concurrent increase in microbial N (Cone *et al.*, 1997), suggesting that the fungal treated straw may lead to a more efficient microbial N conversion in the rumen.

The selectivity of fungi in degrading lignin, as indicated by the “carbohydrate enrichment” or C/L in the remaining material, was significantly correlated ( $r = 0.64$ ,  $P < 0.001$ ) to the IVGP values. Although the chemical analysis were not carried out for all 32 strains involved in this study, these correlations indicate the potency of the IVGP technique as a tool in screening the best performing fungal strains. One of the limitations of using the Van Soest *et al.* (1991) method to analyze the cell wall composition of the fungal-treated material is that, it does not differentiate cellulose in the substrate from chitin in the fungal biomass (cellulose-like structure with *N*-acetylglucosamine monomers). This is because the hydrolysis of chitin polymers requires a stronger concentrated acid (Einbu and Vårum, 2008). This may lead to an overestimation of the cell wall composition, which can explain the slight increase in cellulose observed for CS6 and PE6-treated wheat straw. Nonetheless, in the context of fungal pretreatment of lignocellulosic biomass, a selective fungus should be defined for its ability to degrade lignin and enrich the biomass with high amounts of available carbohydrates in the end products, i.e. high C/L in the remaining materials. Meanwhile, the increases of CP in fungal-treated straw may erroneously suggest that these fungi could fix atmospheric N, which is not true. The relative enrichment of CP is due to degradation of the cell wall components, leading to a concentration of N content (Van Kuijk *et al.*, 2015a).

The present study also provides fundamental information for breeding purposes. For example, CS1 is one of the strains in the *C. subvermispota* collection that has no clamp connection and, therefore, is a monokaryon (Sonnenberg, personal observation, 2017). Tello *et al.* (2001) showed that this monokaryon produced less biomass in liquid cultures and showed a higher lignin-degrading enzyme activity compared to dikaryons. In our study, the low growth rate in the monokaryotic CS1 resulted in a high C/L, which

corresponds to the higher IVGP of the treated wheat straw. Considering all these features, it might be worthwhile to recover the constituent nucleus of all dikaryotic strains and evaluate all recovered nuclei as monokaryons in future breeding activities.

Results clearly show the variation among strains within species in terms of growth patterns, fiber degradation capabilities, as well as improving the *in vitro* digestibility of wheat straw in rumen fluid. Nevertheless, there are some limitations in this screening study. White-rot fungi are known to vary in their performance on different lignocellulosic biomasses (Tuyen *et al.*, 2013). Hence, the selected strains in this study will not necessarily be applicable for other agricultural biomasses. The wheat straw used in the study was heterogeneous in nature, which consisted of different parts of the plant. No prior separation of leaves and stalks was carried out, which may lead to variation in the chemical composition of the straw used. Nevertheless, thorough mixing was carefully done for each sub-batch of the straw to avoid biases. The variation in the wheat straw used, i.e. different cultivars and maturity stages with different culture conditions, may also influence the outcome of future studies using the selected fungal strains. In addition, under various treatment and environmental conditions, these fungi can differ in their enzyme activities and also the production of diverse secondary metabolites, which may affect the subsequent enrichment of the biomass and its degradability by the rumen microbes. This study presents a promising step towards capitalizing on the unique abilities of white-rot fungi to bio-transform high lignin-containing agricultural biomasses into digestible animal feed. Moreover, this finding could be further broadened into various applications such as in the production of biofuels and industrial bioremediation.

## CONCLUSION

In conclusion, there is a large biological variation among fungal strains within the same species for their capacity to degrade lignin in wheat straw. *C. subvermispora* strains, especially strain 1, showed an overall high potential in improving the nutritive value of wheat straw, followed by *L. edodes* and *P. eryngii* strains. The outcome of this study underlines the importance to also assess different strains of the same species and select the best strain for bioprocessing of biomass into ruminant feed. The variation among fungal strains offers opportunities for breeders to map genomic regions that explain the difference in extent and selectivity of lignocellulose degradation. This knowledge can be used to stack favorable alleles and generate superior strains.

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“

A fool thinks himself to be wise,  
but a wise man knows himself  
to be a fool.

”

- WILLIAM SHAKESPEARE

# Variation in the solubilization of crude protein in wheat straw by different white-rot fungi

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**ABSTRACT**

Besides their unique ability to depolymerize cell wall components, white-rot fungi are known to assimilate nitrogenous compounds from substrates. This modification may change protein solubility and fermentation in the rumen. To investigate this, the crude protein (CP) in fungal treated wheat straw (3 fungal species, 2 strains each) was fractionated according to the Cornell Net Carbohydrate and Protein System (CNCPS) and assessed for *in vitro* protein fermentation using a modified gas production technique (IVGP<sub>N</sub>). Results showed that fungi increased fraction A (instantaneously soluble CP; ~2.6 times) and B<sub>1</sub> (rapidly degradable; ~1.2 times); and decreased the slowly degradable fraction B<sub>3</sub> (~41.6%) and unavailable fraction C (~48.3%). The IVGP<sub>N</sub> of straw treated with *Ceriporiopsis subvermispora* strains were not different to the control, but increased by 30.2 to 47.1% in *Pleurotus eryngii* and *Lentinula edodes* strains. The IVGP<sub>N</sub> was significantly ( $P < 0.01$ ) correlated to all fractions of CP, except fraction B<sub>1</sub> and B<sub>2</sub> (intermediately degradable). All fungi also increased the arginine (~56%) and lysine (~15%) contents. This study shows the importance of assessing the protein solubilization by different fungal strains, which can uncover unique mechanisms in the cell wall depolymerization.

**Keywords:** White-rot fungi; different strains/species; protein fractionation; *in vitro* gas production; wheat straw; ruminant feed.

## INTRODUCTION

In recent years, white-rot fungi such as *Ceriporiopsis subvermispota* and *Lentinula edodes*, have been studied for their ability to improve the degradability of agricultural biomass in ruminants (Tuyen *et al.*, 2013; Van Kuijk *et al.*, 2015a). These reports demonstrate clearly that these fungi are able to modify cell wall properties by selectively degrading lignin and increase the amount of potentially fermentable structural carbohydrates. However, little attention has been paid to the effect of the fungal pretreatment on other nutrients in the biomass, particularly protein. Literature shows an increase of total protein content in fungal-treated biomass (Arora and Sharma, 2011; Sharma and Arora, 2010). Although the protein content in biomass such as wheat straw is low (~16.1 g kg<sup>-1</sup> on dry matter basis) (Nayan *et al.*, 2018), the knowledge on fungal modification of protein availability and solubility is useful and can be applied on various biomasses and even forages. Fungi are known for their capabilities in assimilating nitrogen (N) from the substrate (Davis and Wong, 2010). Although ammonium and glutamine are their preferred nitrogenous compounds, fungi can also use N from various other sources, such as nitrate, urea and amines, to synthesize protein (Tudzynski, 2014). We hypothesize that the modification and assimilation of nitrogenous compounds by fungi may change the solubility and availability of protein in the wheat straw for rumen microbes.

Rumen microbes are able to degrade protein from the feed or directly use ammonia and other non-protein nitrogen (NPN) compounds to synthesize microbial N. Utilization of protein from the feed, however, depends on varying proportions of its soluble and insoluble fractions. Different fractions of crude protein in feed have been estimated using the Cornell Net Carbohydrate and Protein System (CNCPS) (Licitra *et al.*, 1996; Sniffen *et al.*, 1992). This method allows partitioning of feed protein into five fractions: Non-protein nitrogen (NPN; fraction A), available true protein (fraction B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>) and unavailable protein (fraction C), which are different in their inherent rates of degradation in the rumen. Fraction A and B<sub>1</sub> are rapidly degraded, while fraction B<sub>2</sub> is fermented at lower rates and can escape the rumen. Fraction B<sub>3</sub> is degraded more slowly due to its association with cell walls and a large proportion escapes the rumen. Fraction C is highly resistant to breakdown in the rumen.

Characterization of the rumen fermentability of the protein fraction in fungal-treated wheat straw has hitherto not been studied. There are a number of *in vitro* techniques to estimate rumen protein fermentation, including enzymatic techniques (Aufrère *et al.*, 1991; Cone *et al.*, 2004). A modified *in vitro* gas production technique has also been used

to estimate protein fermentation characteristics in the rumen (Cone *et al.*, 2009) and large intestine of pigs (Cone *et al.*, 2005). Cone *et al.* (2009) concluded that there was a good relationship ( $R^2=0.85$ ) between the gas production data and the amount of rumen escape protein determined with the nylon bag technique. In brief, the modified technique involves a pre-incubation of diluted rumen fluid with rapidly fermentable carbohydrates in a N-free buffer medium. The approach ensures N to be the limiting factor for microbial growth, so that the subsequent fermentation (and gas production) depend on the availability of N in the samples.

To test the above-mentioned hypothesis, this study aimed to: (1) study the solubilization of the crude protein content, using the Net Carbohydrate and Protein System; (2) evaluate the protein fermentation characteristics, using a modified gas production technique as described by Cone *et al.* (2009); and (3) determine the changes in the amino acid composition of the wheat straw, treated with different fungal strains.

## MATERIALS AND METHODS

### Preparation of the fungal-treated wheat straw

The fungal-treated samples used in the present study were part of a previous experiment (Nayan *et al.*, 2018) where selection of the best performing fungal strains to improve the nutritive value of wheat straw was studied. Two high potential strains from three different fungal species, based on *in vitro* degradability in rumen fluid, were selected for the present study: CS1 (CBS 347.63) and CS12 (ME-485) strains of *Ceriporiopsis subvermispora*; PE3 (Mycelia2600) and PE6 (AL04) of *Pleurotus eryngii* and LE8 (sh 03/08) and LE10 (LE75) of *Lentinula edodes*. A detailed procedure for fungal strain preparation and pretreatment of the wheat straw has been previously described (Nayan *et al.*, 2017). In brief, all strains were grown on malt extract agar and a part of the fungal colony was used as an inoculum to prepare the spawn for each fungus on autoclaved sorghum grains. The inoculated grains was incubated at 24°C until a complete colonization for 4 to 5 weeks. Wheat straw was chopped into approximately 3 cm pieces and soaked in water for 3 days at room temperature. After draining the excess water, the straw was distributed into 2.1 l micropropagation containers (Combiness, Nevele, Belgium). After autoclaving at 121°C for 1 h, the straw was inoculated with the prepared spawn at 10% of the dry weight of the straw in the container. The wheat straw was incubated in triplicate at 24°C for 7 weeks in a climate-controlled chamber. After the



incubation, all samples were freeze-dried and ground over a 1 mm sieve, using a cross beater mill (100AN, Peppink, Olst, the Netherlands).

### Crude protein fractionation

Crude protein (CP) fractionation was performed according to procedures described by Licitra *et al.* (1996), with modifications. Samples were analyzed for total N by Kjeldahl analysis (ISO 5983, 2005) and crude protein was calculated as  $N \times 6.25$ . The non-protein nitrogen (NPN) was obtained by precipitation of true protein in the sample with 10% (w/v) trichloroacetic acid (TCA) for 30 min. After washing 2 times with TCA, the N content in the residues was determined and the NPN was calculated by subtracting residual N from total N. The soluble N was determined by incubating the samples with phosphate-borate buffer (containing  $13.79 \text{ g l}^{-1} \text{ NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and  $8.91 \text{ g l}^{-1} \text{ Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  at pH 6.8) at  $37^\circ\text{C}$  for 3 h. After incubation, the samples were filtered (Whatman 541, Maidstone, UK) and residual N was determined to yield the insoluble N fraction. The CP contents were presented as absolute amounts, i.e. g per 100 g of starting organic matter (OM). The absolute amount was calculated from the remaining amount (g) of freeze-dried sample, which was corrected for the dry matter content. Neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) were determined by N analysis of the neutral detergent fiber (NDF) and acid detergent fiber (ADF) residues (Van Soest *et al.*, 1991), respectively. All five fractions in Cornell Net Carbohydrate and Protein System (CNCPS) were expressed as a percentage of total CP and were calculated according to Sniffen *et al.* (1992). These fractions are illustrated in Fig. 4.1.

### Modified *in vitro* gas production

The modified *in vitro* gas production technique (IVGP<sub>N</sub>) was performed according to the procedures described by Cone *et al.* (2009). Rumen fluid was collected from non-lactating cows, fed concentrate and grass silage *ad libitum*. The N-free buffer/mineral solution contained  $10.03 \text{ g l}^{-1} \text{ NaHCO}_3$ ,  $1.43 \text{ g l}^{-1} \text{ Na}_2\text{HPO}_4$ ,  $1.55 \text{ g l}^{-1} \text{ KH}_2\text{PO}_4$ ,  $0.15 \text{ g l}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $0.52 \text{ g l}^{-1} \text{ Na}_2\text{S}$ ,  $0.017 \text{ g l}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $0.015 \text{ g l}^{-1} \text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $0.002 \text{ g l}^{-1} \text{ CoCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $0.012 \text{ g l}^{-1} \text{ FeCl}_3 \cdot 6\text{H}_2\text{O}$  and  $0.125 \text{ mg l}^{-1}$  resazurin. The rumen fluid was diluted 1:19 with buffer/mineral solution to minimize the N input from the rumen fluid. The buffered rumen fluid was pre-incubated with  $10 \text{ g l}^{-1}$  rapidly fermentable carbohydrates, consisting of  $3.33 \text{ g l}^{-1}$  glucose,  $3.33 \text{ g l}^{-1}$  xylose and  $3.33 \text{ g l}^{-1}$  soluble starch,

TOTAL PROTEIN				
Buffer Soluble P		Buffer Insoluble P		
		NDIP		
			ADIP	
NPN		True Protein		
A	B <sub>1</sub>	B <sub>2</sub>	B <sub>3</sub>	C

**Fig. 4.1.** Fractionation of total crude protein, based on Sniffen *et al.* (1992). P: protein; NDIP: neutral detergent insoluble protein; ADIP: acid detergent insoluble protein; NPN: non-protein nitrogen.

for 5 h at 37°C in a 5 l bottle, under continuous flushing with CO<sub>2</sub>. During this pre-incubation, all available N from the rumen fluid was incorporated into bacterial N components in order to make N limiting for microbial growth. The pre-incubation was also carried out in four 250-ml bottles connected to the gas production equipment for recording the gas production data. After 5 h of pre-incubation, 60 ml of buffered rumen fluid, together with the excess carbohydrates, was added to the bottles with pre-weighed samples containing exactly 5 mg of N (sample DM weight ranged from 1.4 to 2.2 g). Samples were incubated for 72 h and the gas production data were registered automatically. At the end of the incubation, a sample of rumen fluid from each bottle (600 µl) was mixed with 10% trichloroacetic acid (TCA) to determine the ammonia-N (NH<sub>3</sub>-N) content spectrophotometrically at 623 nm. The kinetic parameters were determined by fitting the gas production data to a monophasic model (Groot *et al.*, 1996).

### Chemical composition and amino acids determination

Samples were dried for 4 h in an oven at 103°C to determine the dry matter (DM) content (ISO 6496, 1999) with ash content determined after combustion at 550°C for 3 h in a muffle furnace (ISO 5984, 2002). The fiber contents – exclusive of residual ash, were determined using Van Soest *et al.* (1991), with modification. Neutral detergent fiber (aNDFom) was determined using a heat-stable amylase (thermamy) and alcalase. Acid

detergent fiber (ADFom) and acid detergent lignin (solubilize cellulose with sulphuric acid; lignin-(sa)), were also determined using the standard method. Amino acids were analyzed using the standard AOAC method (AOAC, 2000). The protein in the samples were hydrolyzed with 6 M hydrochloric acids before the individual amino acid composition was separated, detected and quantified using high performance liquid chromatography (HPLC). Tryptophan was not determined. No corrections were made for amino acid loss during hydrolysis, and amino acid weights were calculated using free amino acid molecular weights.

### Statistics

Data were analyzed by two-way analysis of variance using the general linear model in SAS 9.3, followed by post-hoc multiple comparison using least significance differences. The statistical model used was as follows:

$$Y_{ijk} = \mu + SP_i + ST_{j(i)} + \tau_{k(i,j)} + \varepsilon_{ijk}$$

where  $Y_{ijk}$  = response variable  $ijk$ ,  $\mu$  = overall mean,  $SP_i$  = the effect of species  $i$ ,  $ST_{j(i)}$  = the effect of strain  $j$  nested within species  $i$ ,  $\tau_{k(i,j)}$  = effect of week  $k$ , and  $\varepsilon_{ijk}$  = residual error with a mean of 0 and variance  $\sigma^2$ .  $SP_i$  was considered a fixed effect,  $ST_{j(i)}$  and  $\tau_{k(i,j)}$  as random effects. Probability values below 5% were considered significant. Pearson Product-Moment Correlation ( $r$ ) coefficients were also determined among the measured variables.

## RESULTS

### Chemical composition and fractionation of crude protein

Table 4.1 summarizes the chemical composition and the fractionation of CP in wheat straw, treated with different fungal strains. The untreated, autoclaved wheat straw contained  $87.2 \pm 0.3$  g of organic matter (g) per container. All CP fractionation data are presented per 100 g of the starting OM. Fungal pretreatment caused a 1.6 to 7.3% decrease in the total amount of OM by the end of the colonization weeks. The fiber contents were noticeably lower in all fungal-treated wheat straws, particularly the lignin-(sa) content which were decreased by 48.5% in CS1-treated straw. After 7 weeks of colonization, the total amount of CP when expressed per unit starting OM was significantly ( $P < 0.001$ ) increased by 23.3 to 30.9% in the fungal-treated wheat straw, compared to the control. The amount of true protein was lower in straw treated with *P. eryngii* and *L. edodes*

**Table 4.1.** Chemical composition and crude protein fractions of wheat straw, treated with different fungal strains for 7 weeks, in comparison to untreated straw (control).

Parameters	Control		CS strain			PE strain			LE strain			RMSE		Pvalue	
	1	12	1	3	6	8	10	10	10	10	10	Sp	St	Time	
DM (g kg <sup>-1</sup> )	213.9 <sup>c</sup>	209.1 <sup>bc</sup>	199.9 <sup>a</sup>	213.6 <sup>bc</sup>	209.8 <sup>c</sup>	199.7 <sup>a</sup>	201.8 <sup>a</sup>	201.8 <sup>a</sup>	201.8 <sup>a</sup>	201.8 <sup>a</sup>	201.8 <sup>a</sup>	2.90	<0.001	0.017	<0.001
Chemical composition (g kg <sup>-1</sup> DM)															
aNDFom	871.7 <sup>f</sup>	761.8 <sup>b</sup>	701.2 <sup>a</sup>	803.9 <sup>d</sup>	828.0 <sup>c</sup>	772.7 <sup>b</sup>	790.0 <sup>c</sup>	790.0 <sup>c</sup>	790.0 <sup>c</sup>	790.0 <sup>c</sup>	790.0 <sup>c</sup>	6.94	<0.001	<0.001	<0.001
ADFom	585.7 <sup>c</sup>	563.6 <sup>b</sup>	544.7 <sup>a</sup>	571.3 <sup>b</sup>	589.2 <sup>c</sup>	590.8 <sup>c</sup>	587.4 <sup>c</sup>	587.4 <sup>c</sup>	587.4 <sup>c</sup>	587.4 <sup>c</sup>	587.4 <sup>c</sup>	4.76	<0.001	0.004	<0.001
Lignin-(sa)	83.9 <sup>d</sup>	62.3 <sup>c</sup>	43.2 <sup>a</sup>	63.7 <sup>c</sup>	65.3 <sup>c</sup>	54.6 <sup>b</sup>	60.7 <sup>c</sup>	60.7 <sup>c</sup>	60.7 <sup>c</sup>	60.7 <sup>c</sup>	60.7 <sup>c</sup>	2.97	<0.001	0.150	<0.001
CP	16.1 <sup>a</sup>	20.1 <sup>ab</sup>	22.3 <sup>b</sup>	21.9 <sup>b</sup>	20.5 <sup>ab</sup>	21.6 <sup>b</sup>	22.5 <sup>b</sup>	22.5 <sup>b</sup>	22.5 <sup>b</sup>	22.5 <sup>b</sup>	22.5 <sup>b</sup>	2.87	0.034	0.235	0.017
Ash	33.3 <sup>a</sup>	40.6 <sup>cd</sup>	42.5 <sup>c</sup>	41.3 <sup>de</sup>	39.5 <sup>bc</sup>	39.0 <sup>b</sup>	39.5 <sup>bc</sup>	39.5 <sup>bc</sup>	39.5 <sup>bc</sup>	39.5 <sup>bc</sup>	39.5 <sup>bc</sup>	0.80	<0.001	0.052	<0.001
Amount of protein (g/100 g of starting OM) <sup>†</sup>															
Total CP	1.7 <sup>a</sup>	2.2 <sup>b</sup>	2.2 <sup>b</sup>	2.2 <sup>b</sup>	2.1 <sup>b</sup>	2.1 <sup>b</sup>	2.2 <sup>b</sup>	2.2 <sup>b</sup>	2.2 <sup>b</sup>	2.2 <sup>b</sup>	2.2 <sup>b</sup>	0.07	<0.001	0.001	<0.001
True protein	1.5 <sup>bc</sup>	1.7 <sup>d</sup>	1.5 <sup>c</sup>	1.5 <sup>c</sup>	1.3 <sup>a</sup>	1.4 <sup>ab</sup>	1.5 <sup>bc</sup>	1.5 <sup>bc</sup>	1.5 <sup>bc</sup>	1.5 <sup>bc</sup>	1.5 <sup>bc</sup>	0.07	<0.001	<0.001	<0.001
Non-protein nitrogen	0.2 <sup>a</sup>	0.4 <sup>b</sup>	0.4 <sup>b</sup>	0.6 <sup>c</sup>	0.8 <sup>d</sup>	0.7 <sup>cd</sup>	0.7 <sup>cd</sup>	0.7 <sup>cd</sup>	0.7 <sup>cd</sup>	0.7 <sup>cd</sup>	0.7 <sup>cd</sup>	0.08	<0.001	0.124	<0.001
Buffer insoluble protein	1.4 <sup>de</sup>	1.6 <sup>f</sup>	1.5 <sup>ef</sup>	1.4 <sup>cd</sup>	1.2 <sup>a</sup>	1.2 <sup>ab</sup>	1.3 <sup>bc</sup>	1.3 <sup>bc</sup>	1.3 <sup>bc</sup>	1.3 <sup>bc</sup>	1.3 <sup>bc</sup>	0.07	<0.001	0.001	<0.001
Buffer soluble protein	0.2 <sup>a</sup>	0.7 <sup>c</sup>	0.7 <sup>c</sup>	0.8 <sup>d</sup>	0.9 <sup>d</sup>	0.9 <sup>d</sup>	0.9 <sup>d</sup>	0.9 <sup>d</sup>	0.9 <sup>d</sup>	0.9 <sup>d</sup>	0.9 <sup>d</sup>	0.08	<0.001	0.058	<0.001
NDIP	1.2 <sup>c</sup>	1.0 <sup>b</sup>	0.7 <sup>a</sup>	0.9 <sup>b</sup>	0.7 <sup>a</sup>	0.7 <sup>a</sup>	0.7 <sup>a</sup>	0.7 <sup>a</sup>	0.7 <sup>a</sup>	0.7 <sup>a</sup>	0.7 <sup>a</sup>	0.06	<0.001	<0.001	<0.001
ADIP	0.8 <sup>d</sup>	0.7 <sup>c</sup>	0.5 <sup>a</sup>	0.6 <sup>bc</sup>	0.5 <sup>ab</sup>	0.5 <sup>ab</sup>	0.6 <sup>bc</sup>	0.6 <sup>bc</sup>	0.6 <sup>bc</sup>	0.6 <sup>bc</sup>	0.6 <sup>bc</sup>	0.07	<0.001	<0.001	<0.001
Protein fractions (% CP) *															
A	10.5 <sup>a</sup>	15.2 <sup>ab</sup>	20.6 <sup>b</sup>	29.2 <sup>c</sup>	37.7 <sup>d</sup>	32.2 <sup>c</sup>	31.0 <sup>c</sup>	31.0 <sup>c</sup>	31.0 <sup>c</sup>	31.0 <sup>c</sup>	31.0 <sup>c</sup>	3.20	<0.001	0.010	<0.001
B <sub>1</sub>	4.4 <sup>a</sup>	9.0 <sup>ab</sup>	9.6 <sup>ab</sup>	9.0 <sup>ab</sup>	6.6 <sup>ab</sup>	9.2 <sup>ab</sup>	11.0 <sup>b</sup>	11.0 <sup>b</sup>	11.0 <sup>b</sup>	11.0 <sup>b</sup>	11.0 <sup>b</sup>	3.52	0.508	0.037	<0.001
B <sub>2</sub>	15.2 <sup>a</sup>	27.3 <sup>c</sup>	36.1 <sup>d</sup>	19.6 <sup>ab</sup>	20.2 <sup>ab</sup>	27.1 <sup>c</sup>	24.5 <sup>bc</sup>	24.5 <sup>bc</sup>	24.5 <sup>bc</sup>	24.5 <sup>bc</sup>	24.5 <sup>bc</sup>	3.16	<0.001	<0.001	<0.001
B <sub>3</sub>	19.8 <sup>c</sup>	15.7 <sup>bc</sup>	12.4 <sup>ab</sup>	16.0 <sup>bc</sup>	10.0 <sup>ab</sup>	8.0 <sup>a</sup>	7.2 <sup>a</sup>	7.2 <sup>a</sup>	7.2 <sup>a</sup>	7.2 <sup>a</sup>	7.2 <sup>a</sup>	3.68	<0.001	0.305	<0.001
C	50.2 <sup>c</sup>	32.7 <sup>b</sup>	21.4 <sup>a</sup>	26.2 <sup>a</sup>	25.6 <sup>a</sup>	23.5 <sup>a</sup>	26.4 <sup>a</sup>	26.4 <sup>a</sup>	26.4 <sup>a</sup>	26.4 <sup>a</sup>	26.4 <sup>a</sup>	3.26	<0.001	<0.001	<0.001

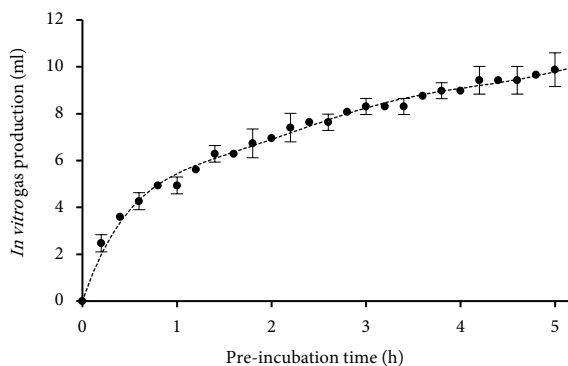
Values with different superscripts within a row are significantly ( $P < 0.05$ ) different. ADFom: acid detergent fiber – exclusive of ash; ADIP: acid detergent insoluble protein; aNDFom: neutral detergent fiber – exclusive of ash; CP: crude protein ( $N \times 6.25$ ); NDIP: neutral detergent insoluble protein; RMSE: Root mean square error; St: strains; Sp: species. <sup>†</sup> Calculated based on the remaining DM (g) after a fungal pretreatment. \* Fractionation of crude protein (as a percentage of CP) based on Sniffen *et al.* (1992).

strains compared to the control, with the true protein content in PE6-treated straw being significantly ( $P = 0.003$ ) lower. CS1 and CS12-treated straw had a significantly ( $P < 0.01$ ) higher amount of true protein, compared to the control. On the other hand, the amount of NPN was significantly ( $P < 0.01$ ) higher using *P. eryngii* and *L. edodes* strains, compared to *C. subvermispota* strains. All fungal-treated wheat straw had a significantly ( $P < 0.001$ ) higher amount of NPN than the control, except for the CS12-treated straw.

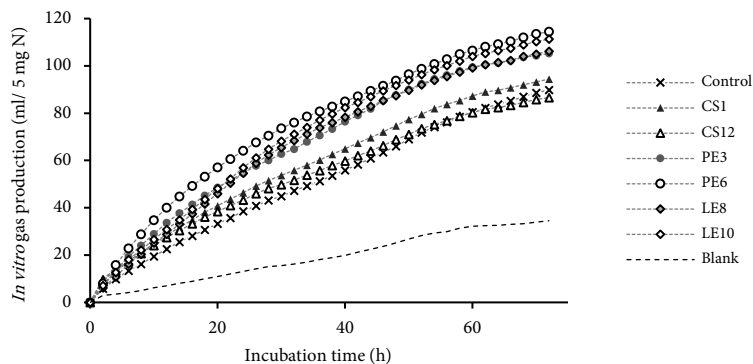
Wheat straw treated with *P. eryngii* and *L. edodes* strains were significantly ( $P < 0.01$ ) lower in the amount of buffer insoluble protein, compared to *C. subvermispota* strains. Contrarily, *P. eryngii* and *L. edodes* strains were higher ( $P < 0.01$ ) in buffer soluble protein than *C. subvermispota* strains. All fungal-treated straw contained a higher ( $P < 0.01$ ) amount of available protein compared to the control. The amount of protein bound to the cell wall was significantly ( $P < 0.05$ ) decreased in fungal-treated wheat straw. The solubilization of the cell wall bound protein resulted in an overall increase of soluble fractions (A and B<sub>1</sub>), while the protein fractions related to cell wall (B<sub>3</sub> and C) were decreased by fungal pretreatment.

### *In vitro* gas production

The IVGP during the 5 h pre-incubation with easily fermentable carbohydrates is shown in Fig. 4.2. A rapid increase of IVGP at a rate of 4.9 ml h<sup>-1</sup> during the first hour was observed. The rate of IVGP then slowed down to 1.35 ml h<sup>-1</sup> from 2 to 4 h and to 0.9 ml h<sup>-1</sup> after 4 h. After 5 h of pre-incubation, the buffered rumen fluid was transferred to the gas production unit and the 72 h incubation with the samples started. The uncorrected gas production profiles for all treatments are also shown in Fig. 4.3. The blanks (N-free



**Fig. 4.2.** *In vitro* gas production during pre-incubation with easily fermentable carbohydrates for 5 h. Error bars indicate standard deviation.



**Fig. 4.3.** *In vitro* gas production for all fungal-treated wheat straw in comparison to untreated wheat straw (control, ×) and a blank, being buffered rumen fluid without sample (-). The gas production profile were not corrected for the blanks.

buffered rumen fluid with the remaining of fermentable carbohydrates) maintained a gas production lower than 10 ml for the first 18 h of incubation. Nonetheless, the gas production continued in blanks and reached a total IVGP of 34.5 ml after 72 h. Ammonia-N ( $\text{NH}_3\text{-N}$ ) was below a detectable amount in all samples of rumen fluid for all treatments after 72 h of incubation.

The corrected gas production profiles ( $\text{IVGP}_N$ ) and their kinetic parameters are summarized in Table 4.2. The  $\text{IVGP}_N$  of wheat straw treated with *P. eryngii* and *L. edodes* strains was significantly ( $P < 0.05$ ) higher than with *C. subvermisporea* strains. Differences in the  $\text{IVGP}_N$  between wheat straw treated with the *C. subvermisporea* strains and the control were not significant. The  $\text{IVGP}_N$  of CS12-treated straw was even lower than that of the control, although not significant. The kinetics data showed an overall better fermentation profile in all fungal-treated wheat straw as indicated by parameters  $B$  (half-time of maximum gas production) and  $R_m$  (maximum fractional rate of degradation) values.

### Changes in amino acids composition

Glutamic acid (Glu), aspartic acid (Asp), histidine (His), alanine (Ala) and glycine (Gly) were the main amino acids (AAs) present in the untreated (control) wheat straw (Table 4.3). Overall, there were no marked changes in the total AA-N content when

**Table 4.2.** Total *in vitro* gas production after 72 h of incubation in buffered rumen fluid and its kinetic parameters for wheat straw treated with different fungal strains for 7 weeks, in comparison to untreated straw (control).

Parameters	Control			CS strain			PE strain			LE strain			RMSE		P value		Time
	1	12		3	6		3	6		8	10		Sp	St			
IVGP <sub>N</sub> (ml/ 5 mg N)	54.3 <sup>a</sup>	59.9 <sup>a</sup>	52.1 <sup>a</sup>	70.8 <sup>b</sup>	79.9 <sup>b</sup>	71.7 <sup>b</sup>	76.8 <sup>b</sup>	5.44	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
Kinetics *																	
<i>B</i>	23.1 <sup>c</sup>	17.6 <sup>ab</sup>	15.9 <sup>ab</sup>	16.6 <sup>ab</sup>	14.8 <sup>a</sup>	18.5 <sup>b</sup>	18.5 <sup>b</sup>	2.01	<0.001	0.062	0.005						
<i>C</i>	1.41 <sup>a</sup>	1.31 <sup>a</sup>	1.23 <sup>a</sup>	1.47 <sup>ab</sup>	1.44 <sup>ab</sup>	1.74 <sup>c</sup>	1.66 <sup>bc</sup>	0.14	<0.001	0.295	0.025						
<i>t</i> <sub>90%</sub>	12.2 <sup>bcd</sup>	7.2 <sup>ab</sup>	4.9 <sup>a</sup>	9.9 <sup>abcd</sup>	8.4 <sup>abc</sup>	15.5 <sup>d</sup>	14.5 <sup>cd</sup>	3.75	<0.001	0.249	0.622						
<i>R</i> <sub>90%</sub>	0.034 <sup>a</sup>	0.044 <sup>b</sup>	0.049 <sup>bc</sup>	0.048 <sup>bc</sup>	0.053 <sup>c</sup>	0.048 <sup>bc</sup>	0.046 <sup>b</sup>	0.004	<0.001	0.025	<0.001						

Values with different superscripts within row are significantly ( $P < 0.05$ ) different. *B*: half time of the maximum gas production (h); *C*: parameters determine curvature of the graph; IVGP<sub>N</sub>: Total *in vitro* gas production (modified technique) corrected for blanks; *t*<sub>90%</sub>: time of the maximum fractional rate of substrate degradation (h); *R*<sub>90%</sub>: maximum fractional rate of substrate degradation (h<sup>-1</sup>); RMSE: Root mean square error; St: strains; Sp: species.

\* Fitted to a monophasic curve (Groot *et al.*, 1996).

**Table 4.3.** Content (g per 100 g CP) of amino acids (AA) in control (untreated) and fungal-treated wheat straw after 7 weeks of colonization.

Parameters	Control			CS strain			PE strain			LE strain			RMSE
	1	12	3	3	6	6	8	8	10	10	10		
<b>Essential AA</b>													
Arg	1.42 <sup>a</sup>	2.68 <sup>d</sup>	1.89 <sup>b</sup>	1.89 <sup>b</sup>	2.22 <sup>bc</sup>	2.36 <sup>cd</sup>	2.09 <sup>bc</sup>	2.09 <sup>bc</sup>	2.09 <sup>bc</sup>	2.09 <sup>bc</sup>	2.09 <sup>bc</sup>	0.21	
His	4.69 <sup>b</sup>	6.08 <sup>c</sup>	2.72 <sup>a</sup>	2.72 <sup>a</sup>	4.61 <sup>b</sup>	2.88 <sup>a</sup>	3.55 <sup>a</sup>	3.55 <sup>a</sup>	3.54 <sup>a</sup>	3.54 <sup>a</sup>	3.54 <sup>a</sup>	0.54	
Ileu	2.70 <sup>bc</sup>	2.71 <sup>bc</sup>	2.37 <sup>a</sup>	2.37 <sup>a</sup>	2.63 <sup>b</sup>	2.29 <sup>a</sup>	2.98 <sup>d</sup>	2.98 <sup>d</sup>	2.87 <sup>cd</sup>	2.87 <sup>cd</sup>	2.87 <sup>cd</sup>	0.14	
Leu	4.14 <sup>abc</sup>	4.51 <sup>bcd</sup>	3.46 <sup>ab</sup>	3.46 <sup>ab</sup>	4.82 <sup>cd</sup>	3.07 <sup>a</sup>	5.71 <sup>d</sup>	5.71 <sup>d</sup>	4.62 <sup>bcd</sup>	4.62 <sup>bcd</sup>	4.62 <sup>bcd</sup>	0.73	
Lys	2.24 <sup>a</sup>	2.41 <sup>ab</sup>	2.58 <sup>bcd</sup>	2.58 <sup>bcd</sup>	2.37 <sup>ab</sup>	2.57 <sup>bcd</sup>	2.79 <sup>d</sup>	2.79 <sup>d</sup>	2.69 <sup>cd</sup>	2.69 <sup>cd</sup>	2.69 <sup>cd</sup>	0.14	
Met	1.68 <sup>bc</sup>	1.23 <sup>ab</sup>	1.09 <sup>a</sup>	1.09 <sup>a</sup>	1.20 <sup>ab</sup>	1.96 <sup>c</sup>	1.62 <sup>abc</sup>	1.62 <sup>abc</sup>	1.38 <sup>ab</sup>	1.38 <sup>ab</sup>	1.38 <sup>ab</sup>	0.32	
Phe	2.05 <sup>ab</sup>	2.04 <sup>ab</sup>	1.87 <sup>a</sup>	1.87 <sup>a</sup>	2.00 <sup>ab</sup>	1.80 <sup>a</sup>	2.55 <sup>b</sup>	2.55 <sup>b</sup>	2.46 <sup>b</sup>	2.46 <sup>b</sup>	2.46 <sup>b</sup>	0.34	
Thr	2.92 <sup>a</sup>	2.94 <sup>a</sup>	2.83 <sup>a</sup>	2.83 <sup>a</sup>	2.83 <sup>a</sup>	3.04 <sup>a</sup>	3.08 <sup>a</sup>	3.08 <sup>a</sup>	3.12 <sup>a</sup>	3.12 <sup>a</sup>	3.12 <sup>a</sup>	0.19	
Val	4.17 <sup>b</sup>	3.79 <sup>a</sup>	3.54 <sup>a</sup>	3.54 <sup>a</sup>	3.87 <sup>ab</sup>	3.50 <sup>a</sup>	3.79 <sup>a</sup>	3.79 <sup>a</sup>	3.85 <sup>ab</sup>	3.85 <sup>ab</sup>	3.85 <sup>ab</sup>	0.22	
<b>Non-essential AA</b>													
Ala	4.59 <sup>c</sup>	4.18 <sup>ab</sup>	4.01 <sup>ab</sup>	4.01 <sup>ab</sup>	4.27 <sup>bc</sup>	3.82 <sup>a</sup>	4.11 <sup>ab</sup>	4.11 <sup>ab</sup>	4.04 <sup>ab</sup>	4.04 <sup>ab</sup>	4.04 <sup>ab</sup>	0.23	
Asp	7.78 <sup>ab</sup>	7.68 <sup>ab</sup>	7.38 <sup>ab</sup>	7.38 <sup>ab</sup>	6.85 <sup>a</sup>	8.00 <sup>b</sup>	7.81 <sup>ab</sup>	7.81 <sup>ab</sup>	7.79 <sup>ab</sup>	7.79 <sup>ab</sup>	7.79 <sup>ab</sup>	0.65	
Cys	2.43 <sup>a</sup>	1.87 <sup>a</sup>	2.06 <sup>a</sup>	2.06 <sup>a</sup>	2.04 <sup>a</sup>	2.51 <sup>a</sup>	2.22 <sup>a</sup>	2.22 <sup>a</sup>	2.37 <sup>a</sup>	2.37 <sup>a</sup>	2.37 <sup>a</sup>	0.38	
Glu	10.81 <sup>ab</sup>	11.04 <sup>ab</sup>	11.82 <sup>ab</sup>	11.82 <sup>ab</sup>	11.97 <sup>b</sup>	10.43 <sup>a</sup>	10.75 <sup>ab</sup>	10.75 <sup>ab</sup>	11.70 <sup>ab</sup>	11.70 <sup>ab</sup>	11.70 <sup>ab</sup>	0.86	
Gly	4.19 <sup>b</sup>	3.47 <sup>a</sup>	3.39 <sup>a</sup>	3.39 <sup>a</sup>	3.37 <sup>a</sup>	3.48 <sup>a</sup>	3.27 <sup>a</sup>	3.27 <sup>a</sup>	3.29 <sup>a</sup>	3.29 <sup>a</sup>	3.29 <sup>a</sup>	0.18	
Pro	3.28 <sup>a</sup>	2.87 <sup>a</sup>	3.04 <sup>a</sup>	3.04 <sup>a</sup>	2.74 <sup>a</sup>	2.98 <sup>a</sup>	3.46 <sup>a</sup>	3.46 <sup>a</sup>	3.40 <sup>a</sup>	3.40 <sup>a</sup>	3.40 <sup>a</sup>	0.65	
Ser	3.39 <sup>a</sup>	3.24 <sup>a</sup>	3.09 <sup>a</sup>	3.09 <sup>a</sup>	3.13 <sup>a</sup>	3.10 <sup>a</sup>	3.41 <sup>a</sup>	3.41 <sup>a</sup>	3.42 <sup>a</sup>	3.42 <sup>a</sup>	3.42 <sup>a</sup>	0.21	
Tyr	1.50 <sup>c</sup>	0.88 <sup>ab</sup>	0.83 <sup>a</sup>	0.83 <sup>a</sup>	0.87 <sup>a</sup>	0.87 <sup>a</sup>	1.26 <sup>bc</sup>	1.26 <sup>bc</sup>	1.12 <sup>abc</sup>	1.12 <sup>abc</sup>	1.12 <sup>abc</sup>	0.23	
<b>Total AA-N*</b>													
DM basis (g kg <sup>-1</sup> DM)	1.56 <sup>a</sup>	2.23 <sup>c</sup>	1.90 <sup>bc</sup>	1.90 <sup>bc</sup>	1.90 <sup>bc</sup>	1.82 <sup>b</sup>	2.06 <sup>abc</sup>	2.06 <sup>abc</sup>	2.13 <sup>de</sup>	2.13 <sup>de</sup>	2.13 <sup>de</sup>	0.12	
Fraction from total N	0.60 <sup>bc</sup>	0.62 <sup>c</sup>	0.54 <sup>a</sup>	0.54 <sup>a</sup>	0.59 <sup>abc</sup>	0.55 <sup>ab</sup>	0.60 <sup>bc</sup>	0.60 <sup>bc</sup>	0.59 <sup>abc</sup>	0.59 <sup>abc</sup>	0.59 <sup>abc</sup>	0.03	
CP basis (g N/ 100 g CP)	9.58 <sup>bc</sup>	9.98 <sup>c</sup>	8.67 <sup>a</sup>	8.67 <sup>a</sup>	9.43 <sup>abc</sup>	8.85 <sup>ab</sup>	9.53 <sup>bc</sup>	9.53 <sup>bc</sup>	9.49 <sup>abc</sup>	9.49 <sup>abc</sup>	9.49 <sup>abc</sup>	0.49	

Values with different superscripts within row are significantly ( $P < 0.05$ ) different. RMSE: Root mean square error; Relative changes of AA contents from control are indicated: More than 50% increase (+++), 20 to 50% increase (++) , 0 to 20% increase (+), less than 20% decrease (-) and more than 20% decrease (- - -).  
 \* Calculated as the sum of the total moles times number of N atoms in amino acids multiplied by 14.



expressed on a CP basis, except for PE3-treated straw which showed a significantly ( $P < 0.05$ ) lower total AA-N content. PE3 significantly ( $P < 0.01$ ) decreased the total essential AAs of the straw by 14.1%. However, all fungi did not cause any significant changes in the non-essential AA content of the straw, although relative decreases were observed in all samples. The changes in the composition of each AA were mostly species-dependent. The strain effect was only significant ( $P < 0.05$ ) for His and methionine (Met). Threonine (Thr), Asp and proline (Pro), however, were not affected by fungal pretreatment. All fungi significantly ( $P < 0.05$ ) increased the arginine (Arg) content of the wheat straw (~56%), but decreased ( $P < 0.001$ ) the Gly content (~19%). Ala was decreased ( $P < 0.05$ ) in wheat straw treated with CS1 (9%), *P. eryngii* (~15%) and *L. edodes* strains (~11%). Glu and Asp contents of the fungal-treated straw were not different to the control.

## DISCUSSION

The present study encompasses two main parts: (1) fractionation of crude protein content in fungal-treated wheat straw; and (2) *in vitro* assessment of protein availability for the fermentation in the rumen. Results show that fungal colonization leads to an increase in the total amount of CP. An increase in the absolute amount of CP suggests an increase of N in the pre-treated straw. This observation needs a further clarification as it is generally accepted that white-rot fungi are unable to fix atmospheric N and only acquire N bound in biomolecules. In nature, fungi utilize fixed atmospheric N through their symbiotic interactions with nitrogen-fixing bacteria and mycorrhizal fungi (Kneip *et al.*, 2007). Since the straw was sterilized, bacteria cannot play a role in N fixation. The increase in the absolute amount of CP can be attributed to the spawn that was prepared on sorghum grains, which contains ~12% of crude protein (Neucere and Sumrell, 1980). The enrichment of the CP content is also due to the expense of other nutrients (Van Kuijk *et al.*, 2015b). Degraded carbohydrates are converted into fungal biomass and into CO<sub>2</sub> leading to a concentration of N content. Due to the quantitative differences in the absolute amounts of CP (control vs. fungal-treated straw), the fractions are expressed on CP basis to allow comparison among different treatments.

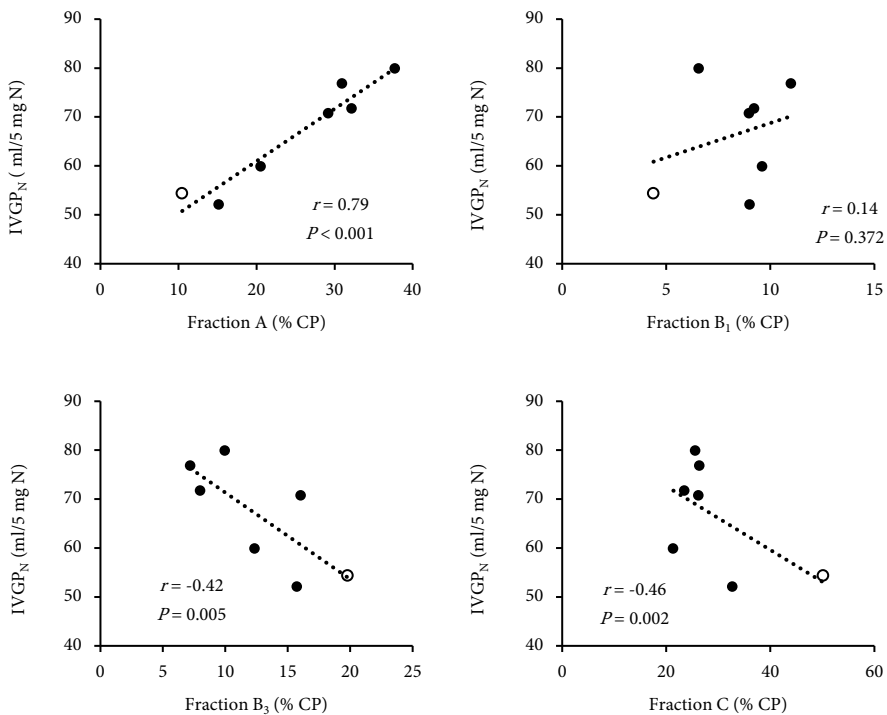
There was no quantitative difference in the total amount of CP among fungal treatments. However, the fractionation of the CP provides a unique insight into the solubilization of protein by different fungal strains. Fungi increased the amount of NPN (fraction A) of the treated straw. The increase of NPN is likely due to the known production of nitrogen-containing secondary metabolites compounds by these higher

fungi, such as nitrogen heterocycles, nucleosides, free amino acids (not bound by peptide bonds) and cyclic peptides (Chen and Liu, 2017). Chitin is also an important NPN component of fungal biomass (Ravi Kumar, 1999). The inclusion of chitin N from fungal biomass in fraction A, however, is disputable. Although trichloroacetic (TCA) has been used as chitin solvent (but at a higher temperature or concentration) (Ravi Kumar, 1999), the amount of N from chitin that is solubilized by TCA in this trial was inferred as low. Chitin can also react with  $\alpha$ -amino acids to give stable complexes (Tharanathan and Kittur, 2003), which would end up in the true protein fraction. We assume that fraction A in this study does not contain chitin and is directly available for the utilization by the rumen microbes. Therefore, wheat straw treated with CS12, all *P. eryngii* and *L. edodes* strains, had a relatively high content of NPN.

All fungi increased the buffer soluble N fractions (A and B<sub>1</sub>) of the wheat straw, especially in *P. eryngii* and *L. edodes* treatments. As expected, there was a decrease in the cell wall bound proteins (fraction B<sub>3</sub> and C). This observation indicates the capability of fungi to liberate more protein associated with the cell wall. Fraction C is likely solubilized by fungi as a consequence of cell wall polymers degradation. There is indeed a clear correlation of a decrease in fraction C to the amount of lignin ( $r = 0.83$ ;  $P < 0.001$ ) and hemicellulose ( $r = 0.76$ ;  $P < 0.001$ ) – the main components degraded by fungi, but not to cellulose. It is worth noting that, cellulose as calculated by subtracting lignin-(sa) from ADFom using the Van Soest *et al.* (1991) method, does not exclude chitin (cellulose-like structure with *N*-acetylglucosamine monomers) from fungal biomass. A complete hydrolysis of chitin requires a stronger concentrated acid (Einbu and Vårum, 2008). Protein compounds that are covalently bound to the cell wall, are partly hydrolyzed in the rumen (Debroas and Blanchart, 1993). Thus, fungal pretreatment can contribute to a higher availability of protein for the rumen microbes.

*In vitro* evaluation of protein fermentation in the rumen provides a complementary finding to the fractionation of protein data. Availability of protein from the gas production was calculated by correcting the values of gas production with blanks. Gas production of the blank is caused by N from the rumen fluid and later on by N released because of microbial turnover (Cone *et al.*, 2009). The incubated samples contained exactly 5 mg N, which made comparison of N availability between the different samples possible. The gas production curves showed a curvilinear to almost linear pattern, indicating a gradual release of N from the samples after the pre-incubation with easily fermentable carbohydrates (Cone *et al.*, 2009). Therefore, a monophasic fit was used, to determine a simple kinetic parameter for the 72 h gas production curve. Results show that

only the  $IVGP_N$  of straw treated with *C. subvermispota* strains were not different to the control, but shows a better kinetic profile, i.e. a higher fractional rate of the substrate degradation ( $R_m$ ). The  $IVGP_N$  was significantly ( $P < 0.01$ ) correlated to all fractions of CP, except fraction  $B_1$  and  $B_2$ . Figure 4.4 shows the correlation plots of  $IVGP_N$  with fraction A,  $B_1$ ,  $B_3$  and C. We also found significant correlations of  $IVGP_N$  to all measured variables, i.e. NPN ( $r = 0.78$ ;  $P < 0.001$ ), buffer soluble protein ( $r = 0.68$ ;  $P < 0.001$ ), NDIP ( $r = -0.49$ ;  $P < 0.001$ ) and ADIP ( $r = -0.44$ ;  $P < 0.001$ ). These observations further support the reliability of using a modified gas production technique in estimating the availability of protein *in vitro* (Cone *et al.*, 2009, 2005). Meanwhile, ammonia-N was below the detection level in the rumen fluid samples, indicating an efficient conversion of available N to microbial N (Cone *et al.*, 1997). This is due to the limited amount of available N in the buffered rumen fluid, with the only N coming from the samples, which is the limiting factor for microbial growth.



**Fig. 4.4.** Correlations among the *in vitro* gas production ( $IVGP_N$ ), soluble protein fractions (A and  $B_1$ ) and cell wall bound protein fractions ( $B_3$  and C) with respective Pearson's  $r$  and  $P$  values. Each point represents mean value for each treatment with 'O' indicates control.

In total, 17 amino acids (AAs) were determined, of which 9 of them are essential for ruminants (Kung and Rode, 1996). All fungi increased the Arg and Lys content of the straw. It is inferred that these AAs are part of the building blocks of fungal protein. These AAs play various roles in the fungal growth and metabolisms, which will not be discussed here. The present study could not provide an accurate view on the roles of each AA in fungi as it requires a more advanced and in-depth study, such as gene expression. An increase in Lys, as one of the limiting AAs in ruminants (Kung and Rode, 1996), is valuable although its total amount in the treated wheat straw may be insignificant. However, fungi did not show a favorable effect on Met, another limiting AA for ruminants. There were variable changes among different fungal strains on other AAs. Some fungi incorporate them in their protein building blocks, while others may use them as a substrate for various biological functions. The total fractions of N in AAs (AA-N) from the total N (from Kjeldahl) showed no significant changes, except a significantly ( $P < 0.05$ ) lower fraction observed for PE3-treated wheat straw. A lower AA-N/total N fraction in most fungal-treated straw also indicates an increase in NPN fraction. Nonetheless, the partitioning of protein in fungal-treated wheat straw needs a further study using a more robust and accurate method, for instance using a  $^{15}\text{N}$ -labeling methods.

The outcome of this study underlines the need to further investigate the ability of fungi to liberate cell wall bound protein and make it more available for the fermentation in the rumen. The relationship between the changes in cell wall polymers to the availability of protein is of great interest. Results indicate a good correlation among them. However, the relationship of available protein with the extent of available carbohydrate “enrichment” (total carbohydrate to lignin ratio in the remaining materials) and *in vitro* degradability of the total OM, remains unclear. For instance, our previous trial showed that CS1 increased the fermentable OM of the straw by ~38%, which is significantly ( $P < 0.001$ ) higher than PE6 treatment (Nayan *et al.*, 2018). However, PE6 had the highest IVGPN and a higher amount of NPN and available protein than CS1. The differences in NDIP and ADIP between the two fungi were not significant. It is certain that a higher fermentability of the total OM is more important compared to the improvement in utilization of protein, although the latter is also of a great interest. In the literature, ligninolytic enzyme activity of *Phanerochaete chrysosporium* is suppressed by a high N concentration, while *Pleurotus ostreatus* shows a high activity (Leatham and Kent Kirk, 1983). Van Kuijk *et al.* (2016) did not find a significant improvement in the extent of lignin degradation and *in vitro* degradability with urea supplementation for *C.*

*subvermispora* and *L. edodes* treatments. Removal of N from hemp with protease improved the selective delignification by *Bjerkandera* sp. (Dorado *et al.*, 2001). These reports clearly indicate a large variation among different fungal species in metabolizing protein and affect their selectivity in lignin degradation. In the comparison between CS1 and PE6, CS1 released most of the protein from the cell wall (fraction B<sub>3</sub> and C), but resulted in a lower protein availability (fraction A and B<sub>1</sub>). Instead, CS1-treated straw showed a higher buffer insoluble fraction (B<sub>2</sub>) compared to PE6. This is probably a mechanism possessed by CS1 to stimulate a higher production of lignin degrading enzymes that may explain its higher selectivity than PE6. Nonetheless, lower protein availability for the fermentation in the rumen is not necessarily a shortcoming as it may also mean that the substrate contains a higher amount of rumen protected protein. This protein can pass through the rumen and may be digested and absorbed in the small intestine of ruminants.

## CONCLUSIONS

Wheat straw treated with *P. eryngii* and *L. edodes* strains resulted in a higher protein availability for fermentation in the rumen. *C. subvermispora* strains on the other hand, did not improve the protein availability. All fungi resulted in a relative enrichment of the protein content. The fungi also increased the arginine and lysine content of the wheat straw. Fractionation of protein and *in vitro* evaluation of protein fermentation in rumen fluid are reliable parameters in assessing protein solubilization by different fungal strains.

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It takes 20 years to build a reputation  
and five minutes to ruin it. If you think  
about that, you'll do things differently.

”

- WARREN BUFFETT



# **The relevance of different analytical methods to assess fungal degradation of lignocellulose and explaining the subsequent ruminal degradability**

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## ABSTRACT

The improved degradability of plant biomass by fungal pretreatment has been attributed to the capability of fungi to modify and degrade the cell wall. We compared two analytical procedures (A: Gravimetric method of Van Soest; and B: Py-GC/MS with monosaccharide analysis) for determining the cell wall contents, in order to explain effects on wheat straw degradability as measured by *in vitro* gas production (IVGP).

Although the lignin to carbohydrate ratio of both procedures showed a significant ( $P < 0.01$ ) negative correlation with the IVGP, Procedure A showed a higher correlation coefficient ( $r$ ) compared to Procedure B (-0.84 vs. -0.69). Procedure A accounts for the residual insoluble cell wall population, particularly acid detergent lignin (ADL), which explains its strong inverse relationship with the IVGP. Nevertheless, Procedure B results in detailed elucidation of remaining lignin and carbohydrate structures, thereby, reflecting the actual modification and degradation of cell wall by fungi. Based on quantitative Py-GC/MS, we showed that IVGP was mainly influenced by lignin content rather than the structural features of lignin. At comparable magnitudes of lignin degradation, differences observed in the remaining lignin structures indicate unique delignification mechanisms of different fungi.

Specific analyses of lignin and carbohydrates contents are required for accurate description of the ruminal degradability of fungal-treated wheat straw. Lignin content rather than its structure, mainly explains the observed degradability.

**Keywords:** White-rot fungi; lignin; carbohydrates; lignin quantification; *in vitro* gas production; Pyrolysis-GC/MS

## INTRODUCTION

Lignocellulosic biomass, such as wheat straw, is used for a broad range of applications including animal feed (Van Kuijk *et al.*, 2015a). Based on routine feed analysis (Van Soest *et al.*, 1991), wheat straw contains (on a dry matter basis, w/w) 46 – 50% cellulose, 26 – 31% hemicellulose and 7 – 9% lignin (acid detergent lignin) (Nayan *et al.*, 2017; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015b). Based on more elaborated analyses, the actual cell wall polymer contents (w/w) are 34 – 43 % cellulose, 26 – 35 % hemicellulosic xylan and 14 – 25% Klason lignin (Kabel *et al.*, 2007; Sun *et al.*, 2004; Szczodrak, 1988). The presence of lignin hinders the enzymatic accessibility to the cell wall polysaccharides, making this biomass a relatively poor source of animal feed, and unsuitable for other applications such as biofuel production (Donkin *et al.*, 2013; Himmel *et al.*, 2007). Unlocking the lignin-carbohydrate complex in the cell wall is a key step in improving the utilization of biomass for various applications. To break the lignin barrier, biomass can be treated physically and/or chemically (Hendriks and Zeeman, 2009; Rabemanolontsoa and Saka, 2016). Due to an increased demand for eco-friendly approaches, biological methods, including the use of white-rot fungi have received much attention in recent years (Nayan *et al.*, 2017; Van Kuijk *et al.*, 2016).

Recent studies reported considerable successes in using fungi such as *Ceriporiopsis subvermispora*, *Lentinula edodes* and *Pleurotus eryngii* for improving the degradability of biomasses in rumen fluid (Nayan *et al.*, 2017; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2017), as determined using the *in vitro* gas production (IVGP) technique (Cone *et al.*, 1996). The IVGP technique is a robust and effective method to assess *in vitro* degradability of substrate in the rumen and has been used as a decisive parameter in selecting high performing fungal strains (Nayan *et al.*, 2018). To assess the relationship of IVGP with changes in the cell wall compositions, routine feed compositional analysis, such as the Van Soest *et al.* (1991) method has been used. Using this gravimetric method, good correlations have been reported for cell wall components – particularly lignin, with IVGP of fungal-treated straw (Nayan *et al.*, 2017; Tuyen *et al.*, 2013; Van Kuijk *et al.*, 2015b). While this gravimetric method is practical and provides a good estimate, it is, however, unspecific. For instance, acid detergent lignin (ADL) – a fraction of the Van Soest *et al.* (1991) method, only represents the recalcitrant lignin content, and does not quantify the acid soluble lignin (Jung *et al.*, 1999). During fungal growth, part of the remaining carbohydrate and lignin may have become soluble and is subsequently removed in the (acid-) soluble fractions, thus further interfere the composition of cell wall, determined by the Van Soest method (Van Kuijk *et al.*, 2016).

The ADL may well correlate with the IVGP as it accounts for the recalcitrant lignin, but provides no information about the remaining lignin structures. The current lack of information regarding exact structures of remaining lignin and carbohydrates limits the understanding of the actual lignin modification by fungi and the subsequent effect on IVGP. As an alternative, semi-quantitative pyrolysis coupled to gas chromatography and mass spectrometry (Py-GC/MS) has been shown to be a powerful tool in estimating the lignin content, while simultaneously providing information on the lignin structural features (Jurak *et al.*, 2015). Recently, lignin quantification by py-GC/MS has been improved by the incorporation of <sup>13</sup>C lignin as internal standard, which allows highly accurate determination of the lignin content (Van Erven *et al.*, 2017). Together with more specific analysis of carbohydrates – by monosaccharides analysis (Englyst and Cummings, 1984), these methods offer a great opportunity for the accurate quantification of lignin and carbohydrate contents, and could provide a better explanation on the changes seen in *in vitro* degradation in rumen fluid, using the IVGP technique.

Recently, high potential strains of *C. subvermispota*, *P. eryngii* and *L. edodes* were identified, based on their ability to improve IVGP of wheat straw (Nayan *et al.*, 2018). These high potential strains showed a high variability in degrading the ADL content of wheat straw (22.3 to 52.2%), which provided a good range of data for the purpose of this study. The aim of this study was to determine the relevance of different analytical methods to measure the cell wall contents in explaining the subsequent changes in the IVGP of wheat straw treated with 6 different fungal strains. To achieve this, two procedures were compared: (1) Procedure A, based on Van Soest *et al.* (1991) method; (2) Procedure B, by combined methods of assessing lignin and carbohydrates, based on Py-GC/MS (Jurak *et al.*, 2015; Van Erven *et al.*, 2017) and monosaccharide analysis (Englyst and Cummings, 1984), respectively.

## MATERIALS AND METHODS

### Preparation of the fungal-treated wheat straw

Two high potential strains from three different fungal species were previously selected based on their ability to improve IVGP of wheat straw: CS1 (CBS 347.63) and CS12 (ME-485) strains of *Ceriporiopsis subvermispota*, PE3 (Mycelia2600) and PE6 (AL04) of *Pleurotus eryngii* and LE8 (sh 03/08) and LE10 (LE75) of *Lentinula edodes* (Nayan *et al.*, 2018). Procedures for the fungal strain preparations and treatment of the wheat straw have been described previously in detail (Nayan *et al.*, 2017). All strains were

maintained on malt extract agar before a piece of that agar ( $1.5 \times 2.0$  cm) was used to prepare the spawn for each fungus. The spawn was prepared using sterilized sorghum grains and was incubated at  $24^{\circ}\text{C}$  for 5 weeks. Organic wheat straw was purchased from a local farmer in the Netherlands, and chopped into approximately 3 cm pieces. The wheat straw was soaked in water for 3 days at room temperature and excess water was drained for 5 h. Adjustments were made based on the final moisture content of the straw ( $\sim 74\%$  w/w), for each container (Combiness, Nevele, Belgium) to contain  $90.2 \pm 0.3$  g of dry matter. After autoclaving at  $121^{\circ}\text{C}$  for 1 h, the straw was inoculated with the prepared spawn at 10% of the dry weight. The wheat straw (treated and untreated with fungi) was incubated in triplicate aerobically at  $24^{\circ}\text{C}$  for 7 weeks in a climate-controlled chamber. All samples were freeze-dried and ground over a 1 mm sieve using a cross beater mill (100AN, Peppink, Olst, the Netherlands).

### ***In vitro* gas production**

The *in vitro* gas production (IVGP) – expressed as  $\text{ml g}^{-1}$  organic matter (OM), was performed according to Cone *et al.* (1996). In brief,  $\sim 0.5 \pm 0.01$  g of sample was incubated in 60 ml buffered rumen fluid at  $39^{\circ}\text{C}$ . The incubation was carried out for 72 h and the gas production was recorded automatically.

### **Estimation of fungal biomass and its ruminal degradability**

Only one strain per fungal species (CS12, PE6 and LE8) was randomly selected for the estimation of fungal biomass and determining the IVGP of its pure mycelial mass. These fungal strains were grown on malt extract agar plates ( $\sim 300$  plates per species), layered with a cellophane on the surface. After 10 days (6 days for CS12), the fully colonized mycelia were harvested. The harvested fresh biomass (3.5 to 6.9 g) was freeze-dried before being subjected to the IVGP and expressed as  $\text{ml g}^{-1}$ . In the IVGP run, the corresponding fungal-treated wheat straws were also included in the same run for comparison. Fungal biomass was estimated with an ergosterol assay, which has been described in detail (Nayan *et al.*, 2018; Niemenmaa *et al.*, 2008). In brief,  $200 \pm 10$  mg sample was extracted with 10% (1:9) KOH/methanol solution, before saponification at  $80^{\circ}\text{C}$  for 60 min. Two series of extractions with distilled water and hexane followed. The collected hexane layers were pooled and dried under vacuum (Rapidvap, Kansas, MO, USA). The extracted ergosterol was re-dissolved in methanol and the solution was filtered before analysis by high performance liquid chromatography (PDA-HPLC) (Alliance HPLC system, Waters, Milford, USA). Cholecalciferol (vitamin  $\text{D}_3$ ) was used as an

internal standard. The ergosterol peak was detected at 280 nm. Previous ergosterol data for wheat straw treated with the selected fungi (Nayan *et al.*, 2018) were used in calculating the conversion factor, CF ( $\text{Ergosterol}_{\text{treated straw}} / \text{Ergosterol}_{\text{pure fungal biomass}}$ ) and estimating the fungal biomass (CF  $\times$  total amount of organic matter, in g) present in the treated straw.

### Conventional feed analyses: 'Van Soest'

Samples were dried in an oven at 103°C to determine the dry matter (DM) content (ISO 6496, 1999); and then further combusted at 550°C for 3 h in a muffle furnace for the ash content (ISO 5984, 2002). Nitrogen content was determined by the Kjeldahl method (ISO 5983, 2005) and crude protein was calculated as  $N \times 6.25$ . Compositional analysis of the fiber contents was carried out according to Van Soest *et al.* (1991), in an ANKOM<sup>200</sup> fiber analyzer (ANKOM Technology, NY). Neutral detergent fiber (NDF) was determined using a heat-stable amylase (thermamyl) and alcalase. Acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined by boiling the sample in an acid detergent solution, and to determine ADL the residue was further treated with 72% v/v H<sub>2</sub>SO<sub>4</sub>. All fiber contents were corrected for ash. Hemicellulose was calculated by subtracting ADF from NDF values, while cellulose was calculated as the difference between ADF and ADL. The cell wall contents were expressed on dry matter basis (% w/w DM). Absolute amounts were calculated from the remaining freeze-dried materials (g), which were corrected for their DM contents. Prior to the monosaccharides and Py-GC/MS analyses, an equal amount of each biological replicate belonging to the same treatment was thoroughly mixed into one sample.

### Carbohydrate content and composition: 'Englyst'

The carbohydrate content and composition was determined in duplicate according to Englyst and Cummings (1984), using inositol as an internal standard. Samples were treated with 72% (w/w) H<sub>2</sub>SO<sub>4</sub> (1 h, 30°C) followed by hydrolysis with 1 M H<sub>2</sub>SO<sub>4</sub> for 3 h at 100°C. The constituent sugars released were analyzed as their alditol-acetates using gas chromatography (ThermoScientific, Waltham, MA, USA) and determined as anhydrocarbohydrates. A standard with a known concentration of glucose, galactose, mannose, arabinose, rhamnose and xylose was taken along in the procedure. The uronic acids released after the acid hydrolysis step, were determined in duplicate as anhydrouronic acid by an automated meta-hydroxydiphenyl assay (Thibault, 1979) with addition of sodium tetraborate using an auto-analyzer (Skalar Analytical BV, Breda, The

Netherlands). Glucuronic acid (Fluka AG, Buchs, Switzerland) was used as a reference (0 – 100  $\mu\text{g ml}^{-1}$ ). Glucan was considered as a total cellulosic polymer, while glucuronoarabinoxylan (GAX) as the hemicellulosic polymer by summing up xylan, arabinan and uronic acid.

### Semi-quantitative and quantitative pyrolysis

Prior to Py-GC/MS, ground wheat straw (1 mm) was ball-milled in a MM200 mixer mill (Retsch, Haan, Germany). Detailed procedures have been described previously (Jurak *et al.*, 2015; Van Erven *et al.*, 2017). Pyrolysis was performed with an EGA/PY-3030D Multi-shot pyrolyzer (Frontier Laboratories, New Ulm, MN, USA) equipped with an AS-1020E Autoshot auto-sampler. The pyrolyzer was coupled to a GC-MS, using a Trace GC, coupled to a DSQ-II mass spectrometer (both Thermo Scientific, Waltham, MA, USA). Pyrolysis of samples (~80  $\mu\text{g}$ ) was performed at 500°C for 1 min. Pyrolysis products were injected into the column via a split/splitless injection (at 250°C) with a splitratio of 1:133 and helium was used as carrier gas. The GC oven was programmed at 270 °C for 15 min. MS detection was used with a scan range of 50-550  $m z^{-1}$  and a scan rate of 4.0 scans  $s^{-1}$ . Compounds were identified by comparing the retention time and mass spectra with standards, the NIST library and published data (Ralph and Hatfield, 1991). For semi-quantitative analysis (samples of week 0, 1, 3 and 7), pyrograms were processed with Xcalibur 2.2 software. Areas were normalized by dividing by corresponding relative response factors (RRF), multiplied with the molecular weight of the respective compound and summed (Van Erven *et al.*, 2017). Lignin content was estimated on the basis of the total area of lignin-derived pyrolysis products and compared to a wheat straw reference sample with known total Klason lignin content (20.5% w/w) (Jurak *et al.*, 2015).

Only samples of week 0 and 7 were subjected to the quantitative Py-GC/MS, using previously described procedures (Van Erven *et al.*, 2017). Briefly, 10  $\mu\text{L}$  of a  $^{13}\text{C}$  lignin internal standard (IS) solution (1  $\text{mg ml}^{-1}$ ) was mixed with ~80  $\mu\text{g}$  of sample and dried before analysis. Lignin-derived pyrolysis products were monitored in the selected ion monitoring (SIM) mode on the two most abundant fragments per compound (both  $^{12}\text{C}$  and  $^{13}\text{C}$ ). The area for each compound was normalized by dividing by the RRF. The RRF values were updated to system performance by recalculation to obtain an identical relative abundance of lignin-derived pyrolysis products of the  $^{13}\text{C}$  IS added to the wheat straw reference sample. Lignin content (% w/w) was determined as the sum of lignin-derived pyrolysis products by the established formula (Van Erven *et al.*, 2017). Relative

abundances of lignin-derived pyrolysis products were based on areas normalized for the  $^{13}\text{C}$  analogues from the IS present in the same sample to distinguish matrix and treatment effects. Areas were not corrected for molecular weight. Compounds were classified according to their structural features (Table 5-S1) and summed. All samples were prepared and analyzed in triplicate.

### Statistics

Data were analyzed using the general linear model (analysis of variance, ANOVA) in SAS 9.3, followed by post-hoc multiple comparison with least significance differences. The statistical model used was as follows:

$$Y_{ijk} = \mu + SP_i + ST_{j(i)} + \tau_{k(i,j)} + \varepsilon_{ijk}$$

where  $Y_{ijk}$  = response variable  $ijk$ ,  $\mu$  = overall mean,  $SP_i$  = the effect of species  $i$ ,  $ST_{j(i)}$  = the effect of strain  $j$  nested within species  $i$ ,  $\tau_{k(i,j)}$  = effect of week  $k$ , and  $\varepsilon_{ijk}$  = residual error with a mean of 0 and variance  $\sigma^2$ .  $SP_i$  was considered a fixed effect,  $ST_{j(i)}$  and  $\tau_{k(i,j)}$  as random effects. Pearson Product-Moment Correlation ( $r$ ) coefficients were also determined among the measured variables. Probability values below 5% were considered significant. No ANOVA was applied on the analytical replicates data of the mixed samples (monosaccharides and Py-GC/MS analyses). However, the coefficient of variation (CV) was calculated as a measure of proportional error (Udén *et al.*, 2012).

## RESULTS AND DISCUSSION

### Cell wall analyses using two procedures and *in vitro* gas production

Changes in the cell wall contents at week 1, 3 and 7, were assessed using two procedures (Fig. 5.1): (1) Procedure A was based on Van Soest *et al.* (1991); and (2) Procedure B represents a more specific analyses using semi-quantitative Py-GC/MS for lignin (Jurak *et al.*, 2015; Van Erven *et al.*, 2017) and carbohydrates were analyzed based on Englyst and Cummings (1984) method. Based on Procedure A, typical weekly changes of the cell wall contents, similar to previous reports, were observed – e.g. relative increases of the ADL contents at week 1, preceded by a large degradation of ADL until the end of colonization week (Nayan *et al.*, 2017; Van Kuijk *et al.*, 2015b). For comparing the two procedures and later discussing their differences in the mass recovery of cell wall components (Fig. 5.1), we focus on the changes in the cell wall composition after 7 weeks of fungal pretreatments (Table 5.1). The cellulose contents, determined by Procedure A,



**Table 5.1.** The *in vitro* gas production (IVGP) and chemical composition, as determined using two procedures, for wheat straw treated with different fungal strains for 7 weeks.

Parameters	Control	<i>C. subvermispورا</i> strain			<i>P. eryngii</i> strain			<i>L. edodes</i> strain			CV
		1	12	3	3	6	8	10	10		
IVGP, ml g <sup>-1</sup> OM	227.7 <sup>a</sup> ± 11.58	313.2 <sup>c</sup> ± 6.56	284.2 <sup>c,d</sup> ± 16.07	252.4 <sup>b</sup> ± 5.47	263.0 <sup>b,c</sup> ± 5.90	297.7 <sup>d,e</sup> ± 10.23	287.3 <sup>d</sup> ± 15.62				5.72
Dry Matter, %	21.4 <sup>b</sup> ± 0.05	20.0 <sup>a</sup> ± 0.01	20.9 <sup>b</sup> ± 0.23	21.0 <sup>b</sup> ± 0.18	21.4 <sup>b</sup> ± 0.11	20.0 <sup>a</sup> ± 0.29	20.2 <sup>a</sup> ± 0.64				1.65
Ash, % w/w DM	3.3 <sup>a</sup> ± 0.04	4.3 <sup>c</sup> ± 0.12	4.1 <sup>c,d</sup> ± 0.07	4.1 <sup>d,e</sup> ± 0.08	4.0 <sup>b,c</sup> ± 0.10	3.9 <sup>b</sup> ± 0.07	4.0 <sup>b,c</sup> ± 0.06				2.10
Compositions, % w/w DM (absolute amount, g)											
CP	1.6 <sup>a</sup> ± 0.03 (1.5)	2.2 <sup>a</sup> ± 0.08 (1.9)	2.0 <sup>a</sup> ± 0.02 (1.8)	2.2 <sup>a</sup> ± 0.09 (1.9)	2.1 <sup>a</sup> ± 0.10 (1.8)	2.2 <sup>a</sup> ± 0.10 (1.8)	2.2 <sup>a</sup> ± 0.02 (1.9)				25.54
Procedure A:											
Cell	50.0 <sup>ab</sup> ± 0.44 (44.0)	50.2 <sup>ab</sup> ± 0.10 (40.5)	49.7 <sup>a</sup> ± 0.92 (42.9)	50.8 <sup>b</sup> ± 0.96 (42.7)	52.4 <sup>c</sup> ± 0.36 (44.5)	53.6 <sup>d</sup> ± 0.10 (43.1)	52.7 <sup>c</sup> ± 0.14 (43.0)				1.05
Hcell	29.1 <sup>c</sup> ± 0.92 (25.6)	15.6 <sup>a</sup> ± 0.66 (12.6)	20.2 <sup>c</sup> ± 0.96 (17.4)	23.3 <sup>d</sup> ± 0.20 (19.6)	23.9 <sup>d</sup> ± 0.43 (20.3)	18.2 <sup>b</sup> ± 0.86 (14.6)	20.3 <sup>c</sup> ± 0.35 (16.5)				3.08
ADL	8.5 <sup>d</sup> ± 0.27 (7.4)	4.3 <sup>a</sup> ± 0.23 (3.5)	6.6 <sup>c</sup> ± 0.81 (5.7)	6.4 <sup>c</sup> ± 0.07 (5.4)	6.5 <sup>c</sup> ± 0.12 (5.6)	5.5 <sup>b</sup> ± 0.33 (4.4)	6.1 <sup>c</sup> ± 0.07 (5.0)				4.75
L/C <sub>A</sub>	0.11 <sup>e</sup> ± 0.003	0.07 <sup>a</sup> ± 0.004	0.10 <sup>d</sup> ± 0.013	0.09 <sup>a</sup> ± 0.001	0.09 <sup>a</sup> ± 0.001	0.08 <sup>b</sup> ± 0.004	0.08 <sup>b,c</sup> ± 0.001				5.44

Table 5.1. (*continue*)

Procedure B:										
Glucan	36.2 ± 0.55 (32.6)	39.8 ± 0.88 (33.6)	41.8 ± 1.53 (37.4)	34.4 ± 1.89 (30.0)	36.9 ± 3.01 (32.5)	39.5 ± 0.36 (33.2)	37.9 ± 2.01 (32.5)	3.48		
GAX	23.9 ± 0.36 (21.6)	20.1 ± 0.65 (17.0)	22.9 ± 1.29 (20.5)	23.6 ± 0.30 (20.6)	24.9 ± 0.83 (21.9)	22.4 ± 0.38 (18.9)	21.9 ± 0.72 (18.7)	4.27		
Lignin <sup>†</sup>	31.5 ± 1.32 (28.4)	8.9 ± 0.42 (7.5)	9.6 ± 0.80 (8.6)	16.7 ± 1.25 (14.6)	16.1 ± 0.25 (14.2)	11.3 ± 0.87 (9.5)	12.6 ± 0.16 (10.6)	4.01		
L/C <sub>B</sub>	0.52 ± 0.022	0.15 ± 0.008	0.15 ± 0.017	0.28 ± 0.019	0.26 ± 0.017	0.18 ± 0.020	0.21 ± 0.006	5.34		

Values with different superscripts within a row are significantly ( $P < 0.05$ ) different. No ANOVA was carried out on Procedure B data and the values are mean of analytical replicates; CV: coefficient of variation; IVGP: total *in vitro* gas production after 72h incubation in the rumen; OM: organic matter; DM: dry matter; CP: crude protein (N × 6.25); Procedure A: Van Soest *et al.* (1991); Cell: cellulose; Hcell: hemicellulose; ADL: acid detergent lignin; L/C<sub>A</sub>: ratio of ADL / (Cell + Hcell); Procedure B: Py-GC/MS and monosaccharide analysis for lignin and carbohydrates, respectively; GAX: glucuronorabinoxylan; L/C<sub>B</sub>: ratio of lignin<sup>†</sup> / (Glucan + GAX).

<sup>†</sup> Lignin content as estimated using semi-quantitative Py-GC/MS.

	Soluble carbohydrates	Plant cell wall contents				Fungal biomass	
		Cellulose	Hemicellulose	Lignin	Other	Glucan	Chitin
<b>Procedure A:<sup>1</sup></b>							
NDF		■	■		■	■	■
ADF		■		■	■		■
ADL				■	■		■
<b>Procedure B:<sup>2</sup></b>							
Englyst	■	■				■	
Py-GC/MS				■			

**Fig. 5.1.** Schematic representation indicating which components are analysed by using two different procedures: (1) Procedure A based on Van Soest *et al.* (1991); cellulose (ADF – ADL), hemicellulose (NDF – ADF); and (2) Procedure B using the Englyst and Cummings (1984) method for carbohydrate analysis and Py-GC/MS for lignin (Van Erven *et al.*, 2017). NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin.

were relatively comparable among different treatments (49.7 to 53.6% DM, w/w). The degradation (absolute amount) of hemicellulose (20.0 to 49.7%) followed a similar pattern to that of ADL (22.3 to 52.2%). Procedure A showed small variations in the ADL content (< 10%) among different biological replicates of the same treatment, while other nutrients showed less than 5% variation. Thereto, the biological replicates were thoroughly mixed to allow analytical triplicates for detailed and more accurate lignin and carbohydrates analyses.

The cellulose content, measured as glucan in Procedure B was 15.8 to 32.2% lower, compared to cellulose measured with Procedure A. Cellulose of Procedure A is overestimated as parts of hemicellulose (e.g. xylan that is associated with lignin) is present in ADF (Fig. 5.1). In addition, a significant portion of lignin is solubilized by the acid detergent solution, leading to overestimation of cellulose contents (Jung, 1997). Similarly, the limitations of ADF (and NDF) lead to inaccuracies in the quantification of hemicellulose. The glucuronoarabinoxylan (GAX) content of untreated straw was 17.8% lower than hemicellulose estimated by the Procedure A. Interestingly, all fungal-treated wheat straw showed 1.5 to 28.5% higher GAX contents, compared to the hemicellulose obtained by Procedure A. It is, however, difficult to deduce a possible explanation for this difference. At week 7, the lignin contents of fungal-treated wheat straw as quantified by

semi-quantitative Py-GC/MS were 1.4 to 2.8 times higher than ADL. Py.lignin of the untreated straw was even higher (3.7 times), compared to ADL. As mentioned before, ADL greatly underestimates the total lignin contents.

For correlations among all measured variables of the two procedures (Table 5.2), all weekly data (excluding control, week 0) were used. Comparing the two procedures, good correlations were only found in the measurement of GAX–hemicellulose ( $r = 0.76$ ;  $P < 0.001$ ) and Py.lignin–ADL ( $r = 0.84$ ;  $P < 0.001$ ). However, in terms of degradation of the absolute amount, Py.lignin gave a higher percentage of degradation (61.9%), compared to the degradation of ADL (33.2%). The degradation percentage of GAX was only 9.3%, compared to 33.4% for hemicellulose. These large differences between procedures indicate disparities in the mass recovery of cell wall components, as proposed in Fig. 5.1. To gain insight, we relate the data with the subsequent effects on ruminal degradability, as measured by IVGP. The changes in IVGP of the fungal-treated straws have been previously discussed in details (Nayan *et al.*, 2018) – hence, will only be mentioned briefly. All fungi clearly increased the IVGP of the wheat straw. Still, large IVGP variations were observed among different species (also to a lesser extent, between strains of the same species), with ~24% difference between CS1 (highest) and PE3 (lowest).

### Relationship between IVGP and the cell wall contents

With Procedure A, the IVGP was positively correlated to cellulose ( $r = 0.46$ ;  $P = 0.070$ ), but negatively correlated to hemicellulose ( $r = -0.84$ ;  $P < 0.001$ ) and ADL ( $r = -0.88$ ;  $P < 0.001$ ). These results are consistent with previous reports, which used the same Procedure A (Nayan *et al.*, 2017; Van Kuijk *et al.*, 2015b). It was reported that the degradation of ADL is accompanied by the solubilization or degradation of hemicellulose (Van Kuijk *et al.*, 2015c). In this study, a significant ( $P < 0.001$ ) correlation of ADL with hemicellulose was observed ( $r = 0.93$ ). The reduction of the hemicellulose content alongside lignin is partly attributed to the fungi's need for growth and metabolisms (Van Kuijk *et al.*, 2015c). The accuracy of Procedure A relies to a large extent on the precision of the extraction and filtration steps (Marichal *et al.*, 2011). Smaller lignin and structural carbohydrates degradation products can easily be excluded from the normal pore size filter bags ( $\Phi = 25 \mu\text{m}$ ). Therefore, the question remains if the changes in the cell wall content are due to 'degradation' or 'solubilization' from the filtration steps. The high correlation of ADL with the IVGP is expected since the ADL fraction may only account for the recalcitrant lignin, which is highly resistant to fermentation in the rumen. This

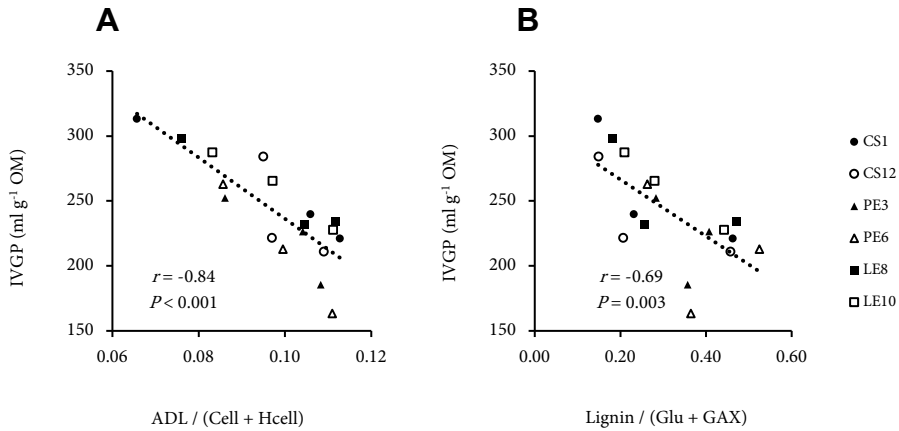
**Table 5.2.** Pearson's correlations coefficient (*r*) among the cell wall contents (% w/w DM), measured using different procedures, to the *in vitro* gas production (IVGP) of the wheat straw treated with different fungal strains for 1, 3 and 7 weeks.

	Procedure A				Procedure B			
	IVGP	Cell	Hcell	ADL	IVGP	Glu	GAX	Py.lignin
IVGP	1				1			
Cell	0.46	1			0.39	1		
Hcell	-0.84***	-0.62**	1		-0.53*	-0.53*	1	
ADL	-0.88***	-0.62**	0.93***	1	-0.73**	-0.65**	0.61*	1

IVGP: total *in vitro* gas production after 72h incubation in the rumen; Procedure A: Van Soest *et al.* (1991); Cell: cellulose; Hcell: hemicellulose; ADL: acid detergent lignin; Procedure B: semi-quantitative Py-GC/MS and monosaccharide analysis for Py.lignin and carbohydrates, respectively; Glu: glucan; GAX: hemicellulosic sugar (glucuronarabinoxylan). Significance of the correlation coefficient (*r*): *P* < 0.05 (\*); *P* < 0.01 (\*\*); *P* < 0.001 (\*\*\*).

recalcitrant lignin covalently bonds to its associated polysaccharides, which lead to a low degradability of cell walls in the rumen (Moore and Jung, 2001). It is inferred that the residual lignin in the ADL fraction may be structurally modified, but is not fragmented by the fungi. To obtain an insight into a possible relationship of the modification of lignin structural features by different fungi with the IVGP, Py-GC/MS was performed – which will be discussed later. Similar circumstances are also applicable to the cellulose contents, where degraded cellulose is solubilized, leaving behind intact cellulose polymers. This explains a low correlation of cellulose with the IVGP.

To compare with the outcome of Procedure A, we assessed the cell wall contents using the specific analyses (Procedure B) and related it with the IVGP. Figure 5.2 shows correlation plots of the IVGP with the ratio of lignin to carbohydrates (L/C), measured with both procedures. The L/C from the two procedures showed significant ( $P < 0.01$ ) relationships with the IVGP, although Procedure A showed a higher correlation coefficient ( $r$ ), compared to Procedure B (-0.84 vs. -0.69, respectively). Both procedures are considered reliable in assessing the IVGP of fungal-treated wheat straw, but there are limitations. Although both procedures show the same trend, the correlation coefficients of the individual cell wall components with the IVGP were noticeably lower for Procedure B. This led to several inferences: (1) Procedure A is more indicative of the cell wall solubility – which explained the IVGP well, rather than indicating the actual cell wall degradation by fungi. The more specific Procedure B elucidates more on the structure, which aids our understanding of variations among fungal strains in modifying the cell wall; (2) Although the recalcitrant lignin (ADL) caused a strong negative effect on the IVGP, the inclusion of all lignin-derived compounds in Py.lignin contents (acid soluble fraction and/or modified lignin fragments) suggested a weaker relationship. The inverse relationship of lignin with digestibility is widely accepted (Moore and Jung, 2001). In their review, Susmel and Stefanon (1993) combined results from several studies, and concluded that an ‘apparent’ digestion of lignin in the rumen (2 to 53%) occurs. However, it is still debatable if modified lignin fragments could be fermented in the rumen and contribute to the IVGP. (3) Procedure B suggests a lesser degradation of hemicellulose, compared to lignin, i.e. both polymers are not simultaneously degraded by fungi, as commonly reported in literature (Nayan *et al.*, 2017; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015b). The fragmented lignin may also contain intact hemicellulose polymers, especially xylan (Buranov and Mazza, 2008), which explains the strong correlation in Procedure A (simultaneous ‘degradation’ of ADL and hemicellulose). This is also a valid explanation to the weaker negative relationship of Py.lignin with IVGP, compared to ADL.



**Fig. 5.2.** Correlations between IVGP to the ratio of lignin to total structural carbohydrates, measured using method A (Van Soest *et al.*, 1991) and B (semi-quantitative Py-GC/MS and alditol-acetates), of wheat straw treated with different fungal strains for 1, 3 and 7 weeks. ADL: acid detergent lignin; Cell: cellulose; Hcell: hemicellulose; Glu: glucan; GAX: glucuronoarabinoxylan. Respective Pearson's  $r$  and  $P$  values are indicated.

During the growth of fungi, the amount of fungal biomass increased in the wheat straw samples. We hypothesize an interference from the fungal biomass with the cellulose and glucan contents (Fig. 1). True (higher) fungi do not contain cellulose, a  $\beta$ -(1 $\rightarrow$ 4)-D-glucan polymer (Badreddine *et al.*, 2008). However, fungal polysaccharides such as  $\beta$ -(1 $\rightarrow$ 3)-D-glucan and  $\alpha$ -(1 $\rightarrow$ 3)-D-glucan (Fesel and Zuccaro, 2016), as well as chitin ( $\beta$ -(1 $\rightarrow$ 4)-linked *N*-acetyl-D-glucosamine), may be included in the total cellulose content of Procedure A. Meanwhile, the acid hydrolysis step in Procedure B will also hydrolyze all glucan belonging to the fungal biomass; but not chitin, which requires a stronger acid (Einbu and Vårum, 2008). Due to its recalcitrant nature, chitin may end up in the ADL fraction. Hence, it can be concluded that none of the two procedures allow accurate quantification of straw cellulose that distinguished from fungal glucan. Besides interfering in the cell wall compositional analyses, the fungal glucan, like the straw cellulose, are fermentable in the rumen (Morgavi *et al.*, 1994), and hence contribute to the total IVGP. Although chitin is thought to be resistant to microbial degradation, several studies reported the presence of chitinolytic microbes and proved the presence of chitinase and *N*-acetyl- $\beta$ -glucosaminidase activities in the rumen (Kopečný *et al.*, 1996; Morgavi *et al.*, 1994). Due to the fact that the IVGP is the cumulative production of CO<sub>2</sub>

(~65%), methane (~31%) and other minor gases (Moate *et al.*, 1997) from any fermentable OM, we also investigated the fermentability of pure fungal biomass in rumen fluid.

### Estimation of fungal biomass and its contribution to the total IVGP

Table 5.3 summarizes the estimation of the fungal biomass (measured as ergosterol) present in the treated wheat straw for one strain of each species after 7 weeks of growth. Results showed that the biomass of CS12 only accounted for 1.7% of the total OM, lower than that of PE6 (2.3%) and LE8 (2.8%). This estimation is in line with the visual observation of these fungi (not shown), where a layer of thin mycelium is a typical growth characteristic of *C. subvermispota* strains. This estimation suggests only a slight contribution of the total fungal biomass to the total weight of OM. However, this rough estimation may not be accurate as the ergosterol content can change with the substrate used (agar plate vs. straw). Furthermore, ergosterol only accounts for less than 3% of the fungal biomass (Klamer and Bååth, 2004), hence, the calculated conversion factors may be misleading. The IVGP of the pure fungal biomass and the corresponding treated wheat straw is shown in Fig. 5.3. The IVGP data were not corrected for ash and, therefore, expressed as ml g<sup>-1</sup> substrate to show patterns. Overall, the pure mycelial mass resulted in a higher IVGP compared to their respective treated straws, by a factor of 1.03 to 1.20. A large variation was observed for CS12 biomass, which is probably due to measurement errors (n = 2). The maximum IVGP observed for CS12 biomass was 301.4 ml g<sup>-1</sup>.

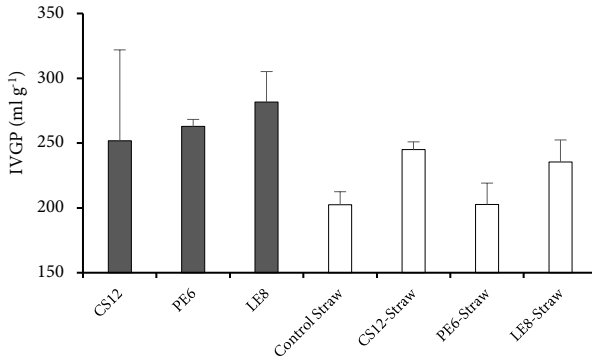
**Table 5.3.** Estimation of fungal biomass present in wheat straw treated with *C. subvermispota* (CS12), *P. eryngii* (PE6) and *L. edodes* (LE8) for 7 weeks.

Strain	Ergosterol (µg g <sup>-1</sup> )		Conversion factor <sup>†</sup>	Estimated fungal biomass in treated straw <sup>‡</sup>	
	Treated straw	Pure fungal biomass		Weight (g)	% in total OM
CS12	59.6 <sup>b</sup>	3466.7 <sup>b</sup>	0.017 <sup>b</sup>	1.4 <sup>b</sup>	1.7 <sup>b</sup>
PE6	64.9 <sup>b</sup>	2821.2 <sup>c</sup>	0.023 <sup>ab</sup>	1.9 <sup>ab</sup>	2.3 <sup>ab</sup>
LE8	112.3 <sup>a</sup>	4049.3 <sup>a</sup>	0.028 <sup>a</sup>	2.3 <sup>a</sup>	2.8 <sup>a</sup>
CV	10.59	7.50	15.28	16.80	15.79

Values with different superscripts within column are significantly ( $P < 0.05$ ) different. CV: coefficient of variation.

<sup>†</sup> Ergosterol<sub>treated straw</sub> / Ergosterol<sub>pure fungal biomass</sub>. <sup>‡</sup> Conversion factor × total amount of organic matter, in g.





**Fig. 5.3.** The *in vitro* gas production (IVGP) of pure fungal biomass (■) and the corresponding wheat straw (□) treated with *C. subvermispura* (CS12), *P. eryngii* (PE6) and *L. edodes* (LE8) for 7 weeks. Error bars indicate standard deviation.

Therefore, we infer that the degradability of pure mycelial mass could have followed the same patterns observed for the respective bioprocessed straw. These results show that the fungal biomass is fermentable in the rumen and may contribute to the total IVGP, although the fermentation of straw polymers is still the major part of the observed IVGP. However, the *in situ* infiltration of fungal biomass in lignocellulose matrix, may affect its effective degradability in the rumen.

Based on the points that have been discussed, we consider lignin as the main determinant of ruminal degradability. Therefore, an in-depth assessment of both lignin content and structures was carried out using a highly accurate quantitative <sup>13</sup>C-IS Py-GC/MS (Van Erven *et al.*, 2017).

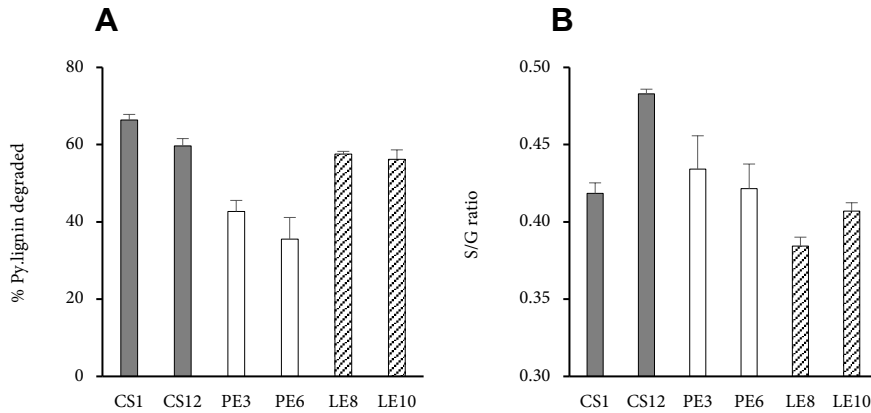
### The lignin structural features and its relationship with the IVGP

The quantitative Py-GC/MS of the samples released 49 lignin-derived compounds (*see* supporting information Table S1), which were used for the determination of Py.lignin<sub>(Q)</sub> contents and structural features (Table 5.4). Based on this method, a similar species-dependent trend in the extent of Py.lignin<sub>(Q)</sub> degradation was observed, with *C. subvermispura* strains degrading most lignin (~63.3%), followed by *L. edodes* strains (~58.0%) and *P. eryngii* (~38.7%). The Py.lignin<sub>(Q)</sub> contents were significantly ( $P < 0.05$ ) correlated to the IVGP ( $r = -0.87$ ). The syringyl to guaiacyl (S/G) ratio, however, did not follow the same patterns seen with the degradation of Py.lignin<sub>(Q)</sub> (Fig. 5.4), suggesting an

**Table 5.4.** Characterization of lignin and its structural moieties in wheat straw treated with different fungal strains for 7 weeks, using quantitative  $^{13}\text{C}$ -IS Py-GC/MS.

Parameters	Control	<i>C. subvermispora</i> strain			<i>P. eryngii</i> strain			<i>L. edodes</i> strain		
		1	12	3	6	8	10			
Py.lignin, % w/w DM (amount, g)	24.0±0.83 (21.6)	8.6±0.36 (7.3)	9.6±0.41 (8.6)	14.2±0.72 (12.4)	16.1±1.07 (14.1)	10.7±0.39 (8.9)	10.9±0.29 (9.2)			
Lignin sub-units, %										
H	9.6±0.40	12.0±0.74	12.1±0.26	10.0±0.71	10.1±0.79	11.1±0.47	11.3±0.46			
G	62.2±0.66	62.1±0.52	59.3±0.29	62.8±1.40	63.3±0.27	64.2±0.24	63.0±0.56			
S	28.2±0.54	26.0±0.42	28.6±0.07	27.2±0.78	26.7±0.93	24.7±0.37	25.6±0.13			
S/G ratio	0.45±0.01	0.42±0.01	0.48±0.00	0.43±0.02	0.42±0.02	0.38±0.01	0.41±0.01			
Structural moieties, %										
Unsubstituted	4.6±0.16	9.4±0.46	9.4±0.37	6.1±0.37	5.8±0.02	7.1±0.35	7.7±0.40			
Methyl	2.0±0.04	3.2±0.28	3.4±0.25	2.6±0.12	2.5±0.24	2.9±0.06	2.9±0.08			
Ethyl	0.2±0.01	0.4±0.04	0.4±0.03	0.3±0.01	0.3±0.03	0.3±0.01	0.3±0.02			
Vinyl	30.6±0.97	30.1±1.65	30.3±0.29	29.6±0.15	31.6±1.18	31.7±0.58	31.3±0.73			
$C_{\alpha}$ -Oxidized	3.7±0.11	13.6±0.64	14.7±0.69	6.0±0.39	5.2±0.33	6.6±0.15	6.9±0.28			
$C_{\beta}$ -Oxidized	1.4±0.02	2.5±0.09	2.7±0.11	1.7±0.05	1.6±0.05	1.8±0.03	1.8±0.04			
$C_{\gamma}$ -Oxidized	54.8±0.92	45.3±2.34	43.9±0.90	52.1±0.65	51.4±1.40	48.8±0.62	48.6±0.57			
Miscellaneous	3.1±0.07	3.2±0.06	3.1±0.05	3.5±0.12	3.2±0.47	3.4±0.06	3.4±0.17			

No ANOVA was carried out on the data and the values are mean of analytical replicates. H: *p*-hydroxyphenyl unit, G: guaiacyl lignin subunit, S: syringyl unit.



**Fig. 5.4.** Degradation (%) of Py.lignin (A), as quantified using Py-GC/MS with  $^{13}\text{C}$  as an internal standard for 7 weeks treatments of wheat straw with *C. subvermispota* (CS1, CS12), *P. eryngii* (PE3, PE6) and *L. edodes* (LE8, LE10). The ratio of syringyl (S) to guaiacyl (G) ratio for each treated straw is shown in Graph B. Error bars indicate standard deviation.

independent relationship between the degradation of Py.lignin<sub>(Q)</sub> contents with the monolignol subunits composition. Previously, Van Kuijk *et al.* (2016) showed a strong correlation ( $r = -0.75$ ) of the S/G ratio with the IVGP for wheat straw treated with a single fungus – *L. edodes*. In the present study, there was a poor correlation of the pooled S/G ratio to the IVGP ( $r = -0.28$ ) and even to the Py.lignin contents ( $r = -0.08$ ), indicating a unique preference of different fungi in attacking certain lignin subunits. Most of the tested fungi here showed only a slight preference in degrading the S-unit compounds (e.g. *trans*-sinapyl alcohol) compared to the G-units, which is a common observation in fungal degradation of lignocellulose (Del Río *et al.*, 2002; Martínez *et al.*, 2011; Van Kuijk *et al.*, 2016). The S-unit compounds have a slightly lower redox potential and fewer aryl-*O*-aryl bonds, making them more susceptible to fungal degradation (Del Río *et al.*, 2002). We also observed a slight tendency of CS12 to decrease the abundances of G-unit compounds (4.7%). These results suggest that the fungal preference on changing S/G was hardly shown, probably due to the extensive degradation of the total Py.lignin<sub>(Q)</sub> contents.

Among the remarkable changes observed in structural moieties of lignin were the increases of unsubstituted (1.2 to 2.0 times) and C <sub>$\alpha$</sub> -oxidized compounds (1.4 to 4.0 times) in fungal-treated wheat straw, compared to the control (Table 5.4). A familiar species-dependent order (*C. subvermispota* > *L. edodes* > *P. eryngii*) for these parameters can be seen. These observations clearly indicate extensive degradation of lignin inter-units,

which resulted in residual lignin with fewer intact linkages. Since the remaining Py.lignin<sub>(Q)</sub> contents of both strains of *C. subvermispota* and *L. edodes* were considered comparable (Fig. 4), only the specific lignin moieties from these fungal-treated straw were used to independently assess their relationships with IVGP. By just considering *C. subvermispota* and *L. edodes*, these specific lignin moieties showed good correlations with the Py.lignin contents, e.g. with substituted ( $r = -0.88$ ) and C $_{\alpha}$ -oxidized ( $r = -0.87$ ), although these values were not significant (Table 5.5). There was also no significant correlation of these moieties with the IVGP. This indicates a fundamental difference among different fungal species in modifying lignin structures that requires further investigation. Among the limitations of this study to correlate structural changes to the subsequent IVGP are the assessment on the inter-unit linkages and the associated carbohydrates. In wheat straw, lignin is covalently bound to structural carbohydrates, via ether and ester-linked *p*-hydroxycinnamic acids (ferulate) (Buranov and Mazza, 2008). Therefore, it is also important to assess fungal modifications of these linkages using a combination of Py-GC/MS with 2D nuclear magnetic resonance (NMR) methods for a better understanding of the underlying delignification mechanisms of different fungi.

**Table 5.5.** Pearson's correlations coefficient ( $r$ ) among the lignin content and selected structural features, determined using quantitative Py-GC/MS, with the *in vitro* gas production (IVGP) of the wheat straw treated with *C. subvermispota* and *L. edodes*.

	IVGP	Py.lignin	Unsub	Methyl	C $_{\alpha}$ -Ox	C $_{\gamma}$ -Ox
IVGP	1					
Py.lignin	-0.62	1				
Unsub	0.20	-0.88	1			
Methyl	0.01	-0.78	0.94	1		
C $_{\alpha}$ -Ox	0.17	-0.87	0.98*	0.98*	1	
C $_{\gamma}$ -Ox	-0.03	0.80	-0.96*	-1.00**	-0.99*	1

IVGP: total *in vitro* gas production after 72h incubation in the rumen; Py.lignin: lignin contents quantified using Py-GC/MS with <sup>13</sup>C lignin internal standard; Unsub: unsubstituted compounds; Methyl: methyl-substituted compounds; C $_{\alpha}$ -ox, C $_{\gamma}$ -ox: compounds with oxidation at C $_{\alpha}$  and C $_{\gamma}$  position, respectively. Significance of the correlation coefficient ( $r$ ):  $P < 0.05$  (\*);  $P < 0.01$  (\*\*).

## CONCLUSION

The “classical” gravimetric and more accurate Py-GC/MS methods to characterize plant cell walls provided a clear difference in the amount of lignin degraded by different fungal strains/species. The gravimetric method underestimates lignin degradation, likely due to the fact that only insoluble (recalcitrant) lignin is measured. Since acid detergent lignin provides a good correlation with degradability, it is likely that this recalcitrant lignin is the main factor for the low degradability of lignocellulose by rumen microorganisms. Fungal biomass interferes with the quantification of cellulose contents – for both investigated methods, and contributes slightly to the total IVGP. Lignin content, rather than its structural features, is the main determinant of degradability when assessing bioprocessing of biomass by different fungal strains.

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Supplementary information for:

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## **The relevance of different analytical methods to assess fungal degradation of lignocellulose and explaining the subsequent ruminal degradability**

Nazri Nayan, Gijs van Erven, Mirjam A. Kabel, Anton S.M. Sonnenberg, Wouter H. Hendriks, and John W. Cone



**Table 5-S1.** Identities and relative abundances of lignin-derived compounds released upon Py-GC/MS of wheat straw treated with different fungal strains for 7 weeks.

Label	Compound	Origin	Ret. Time	MW <sup>12</sup> C	Structure*	Control	<i>C. subvermispora</i> strain			<i>P. eryngii</i> strain			<i>L. edodes</i> strain	
							1	12		3	6	8	10	
1	phenol	H	10.03	94	Unsub	0.7	2.0	1.9	1.1	0.9	1.5	1.6		
2	guaiacol	G	10.31	124	Unsub	2.1	4.2	4.0	2.7	2.5	3.2	3.4		
3	2-methylphenol	H	11.29	108	Methyl	0.2	0.4	0.4	0.2	0.2	0.3	0.3		
4	4-methylphenol	H	12.23	108	Methyl	0.5	1.1	1.2	0.8	0.7	0.9	1.0		
5	4-methylguaiacol	G	13.01	138	Methyl	0.7	1.0	1.0	0.9	0.9	0.9	0.9		
6	2,4-dimethylphenol	H	13.46	122	Methyl	0.1	0.2	0.2	0.1	0.1	0.1	0.2		
7	4-ethylphenol	H	14.52	122	Ethyl	0.1	0.1	0.1	0.1	0.1	0.1	0.1		
8	4-ethylguaiacol	G	15.19	152	Ethyl	0.1	0.2	0.2	0.2	0.2	0.2	0.2		
9	4-vinyguaiacol	G/FA	16.64	150	Vinyl	20.3	20.4	20.3	19.9	21.4	21.7	21.0		
10	4-vinylphenol	H/PCA	16.77	120	Vinyl	7.8	7.7	7.8	7.3	7.8	7.8	7.9		
11	eugenol	G	17.26	164	Misc	0.2	0.2	0.2	0.2	0.2	0.2	0.2		
12	4-propylguaiacol	G	17.34	166	Misc	0.1	0.2	0.2	0.2	0.2	0.2	0.2		
13	syringol	S	18.00	154	Unsub	1.9	3.2	3.5	2.3	2.3	2.4	2.7		
14	<i>cis</i> -isoeugenol	G	18.63	164	Misc	0.1	0.1	0.1	0.1	0.1	0.1	0.1		
15	4-propenylphenol	H	19.54	134	Misc	0.1	0.1	0.1	0.1	0.1	0.1	0.1		
16	<i>trans</i> -isoeugenol	G	19.9	164	Misc	0.8	1.0	0.9	1.1	1.1	1.1	1.1		
17	4-methylsyringol	S	20.26	168	Methyl	0.5	0.6	0.6	0.6	0.6	0.6	0.6		
18	vanillin	G	20.35	152	C <sub>6</sub> -ox	1.2	2.1	2.3	1.4	1.2	1.4	1.4		
19	4-propylnequaiacol	G	20.64	162	Misc	0.1	0.1	0.1	0.1	0.1	0.1	0.1		
20	4-allenequaiacol	G	20.90	162	Misc	0.1	0.1	0.1	0.1	0.1	0.1	0.1		
21	homovanillin	G	21.81	166	C <sub>6</sub> -ox	0.5	0.7	0.8	0.5	0.5	0.5	0.5		
22	4-ethylsyringol	S	22.00	182	Ethyl	0.0	0.0	0.1	0.1	0.0	0.0	0.0		
23	acetovanillone	G	22.27	166	C <sub>6</sub> -ox	0.3	1.1	1.2	0.6	0.5	0.6	0.7		
24	4-hydroxybenzaldehyde	H	23.01	122	C <sub>6</sub> -ox	0.2	0.3	0.3	0.2	0.1	0.2	0.2		
25	4-vinylsyringol	S	23.32	180	Vinyl	2.4	2.0	2.2	2.3	2.4	2.3	2.3		
26	guaiacylacetone	G	23.50	180	C <sub>6</sub> -ox	0.3	0.7	0.7	0.4	0.3	0.4	0.4		

Table 5-S1. (Continued)

27	4-allylsyringol	S	23.75	194	Misc	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
28	propovanillone	G	24.18	180	C <sub>α</sub> -ox	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1
29	guaiacyl vinyl ketone	G	24.47	178	C <sub>α</sub> -ox	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1
30	vanilloyl acetaldehyde	G	24.69	194	C <sub>α</sub> -ox, C <sub>γ</sub> -ox	0.2	4.3	3.9	0.9	0.9	0.6	0.6	4.3	3.9	0.9	0.9	1.5	1.5	1.5
31	<i>cis</i> -4-propenylsyringol	S	24.88	194	Misc	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
32	4-propynesyringol	S	25.52	192	Misc	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
33	4-allenesyringol	S	25.73	192	Misc	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
34	<i>trans</i> -4-propenylsyringol	S	26.19	194	Misc	0.9	0.8	0.9	1.0	0.6	0.6	0.6	0.8	0.9	0.9	0.9	0.9	0.9	0.9
35	dihydroconiferyl alcohol	G	26.22	182	C <sub>γ</sub> -ox	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
36	syringaldehyde	S	26.90	182	C <sub>α</sub> -ox	0.7	0.8	1.0	0.7	0.7	0.7	0.7	0.8	1.0	0.7	0.6	0.6	0.7	0.7
37	<i>cis</i> -coniferyl alcohol	G	26.76	180	C <sub>γ</sub> -ox	1.3	1.1	1.0	1.4	1.3	1.3	1.3	1.1	1.0	1.4	1.3	1.3	1.3	1.3
38	homosyringaldehyde	S	27.75	196	C <sub>β</sub> -ox	0.3	0.4	0.5	0.3	0.3	0.3	0.3	0.4	0.5	0.3	0.3	0.3	0.3	0.3
39	acetosyringone	S	28.20	196	C <sub>α</sub> -ox	0.6	1.2	1.4	0.9	0.6	0.8	0.8	1.2	1.4	0.9	0.8	0.8	0.8	0.8
40	<i>trans</i> -coniferyl alcohol	G	28.55	180	C <sub>γ</sub> -ox	31.6	22.4	20.0	30.0	29.8	29.8	29.8	22.4	20.0	30.0	28.3	28.3	27.7	27.7
41	<i>trans</i> -coniferaldehyde	G	28.92	178	C <sub>γ</sub> -ox	1.8	2.0	1.8	2.1	1.9	1.9	1.9	2.0	1.8	2.1	2.0	2.0	1.9	1.9
42	syringylacetone	S	29.13	210	C <sub>β</sub> -ox	0.3	0.7	0.8	0.4	0.4	0.4	0.4	0.7	0.8	0.4	0.5	0.5	0.5	0.5
43	propiosyringone	S	29.75	210	C <sub>α</sub> -ox	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1
44	syringoyl acetaldehyde	S	29.90	224	C <sub>α</sub> -ox, C <sub>γ</sub> -ox	0.2	3.4	4.0	1.0	0.9	0.9	0.9	3.4	4.0	1.0	1.1	1.1	1.4	1.4
45	syringyl vinyl ketone	S	30.03	208	C <sub>α</sub> -ox	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0
46	dihydrosinapyl alcohol	S	30.7	212	C <sub>γ</sub> -ox	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
47	<i>cis</i> -sinapyl alcohol	S	32.22	210	C <sub>γ</sub> -ox	0.9	0.6	0.6	0.8	0.6	0.8	0.6	0.6	0.6	0.8	0.6	0.7	0.7	0.7
48	<i>trans</i> -sinapyl alcohol	S	33.94	210	C <sub>γ</sub> -ox	16.6	9.3	10.0	13.6	13.7	13.7	13.7	9.3	10.0	13.6	11.6	11.6	11.9	11.9
49	<i>trans</i> -sinapaldehyde	S	34.15	208	C <sub>γ</sub> -ox	2.0	2.3	2.4	2.4	2.3	2.3	2.3	2.3	2.4	2.4	2.1	2.1	2.0	2.0

G: guaiacyl unit, H: *p*-hydroxyphenyl unit, S: syringyl unit, FA: ferulic acid, PCA: *p*-coumaric acid; Ret. Time: Retention time; MW <sup>12</sup>C: Molecular weight (g/mol).\* Unsub: unsubstituted; Methyl, ethyl, vinyl: substituted compounds; Misc: miscellaneous structures; C<sub>α</sub>-ox, C<sub>β</sub>-ox, C<sub>γ</sub>-ox: compounds with oxygen at C<sub>α</sub>, C<sub>β</sub> and C<sub>γ</sub> position, respectively.



“

The weak can never forgive. Forgiveness  
is the attribute of the strong.

”

- MAHATMA GANDHI

# **Mechanistic insight in the selective delignification of wheat straw by three white-rot fungal species through quantitative $^{13}\text{C}$ -IS Py-GC/MS and whole cell wall HSQC-NMR**

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**ABSTRACT**

The white-rot fungi *Ceriporiopsis subvermispota* (CS), *Pleurotus eryngii* (PE) and *Lentinula edodes* (LE) have been shown to be high-potential species for selective delignification of plant biomass. This delignification improved polysaccharide degradability, which currently limits the efficient lignocellulose conversion into biochemicals, biofuels and animal feed. Since selectivity and time-efficiency of fungal delignification still needs optimization, detailed understanding of the underlying mechanisms at molecular level is required. The recently developed methodologies for lignin quantification and characterization now allow the in-depth mapping of fungal modification and degradation of lignin and thereby enable resolving of the underlying mechanisms.

Wheat straw treated by two strains of CS (CS1 and CS12), PE (PE3 and PE6) and LE (LE8 and LE10) was characterized by using semi-quantitative Py-GC/MS during fungal growth (1, 3 and 7 weeks). The remaining lignin after 7 weeks was quantified and characterized by using  $^{13}\text{C}$  lignin internal standard based Py-GC/MS and whole cell wall HSQC NMR. Strains of the same species showed similar patterns of lignin removal and degradation. CS and LE outperformed PE in terms of extent and selectivity of delignification (CS $\geq$ LE $\gg$ PE). The highest lignin removal (66% w/w; CS1) was obtained after 7 weeks, without extensive carbohydrate degradation (factor 3 increased carbohydrate/lignin ratio). Further, though after treatment with CS and LE comparable amounts of lignin remained, the structure of the residual lignin vastly differed. For example,  $\text{C}_\alpha$ -oxidized substructures accumulated in CS-treated lignin by up to 24% of total aromatic lignin, a factor two higher than in LE-treated lignin. Contrarily, ferulic acid substructures were preferentially targeted by LE (and PE). Interestingly, PE-treated lignin was specifically depleted in triclin (40% reduction). The overall subunit composition (H:G:S) was not affected by fungal treatment.

CS and LE are both able to effectively and selectively delignify wheat straw, though the underlying mechanisms are fundamentally different. We are the first to identify that CS degrades the major  $\beta$ -O-4 ether linkage in grass lignin mainly via  $\text{C}_\beta$ -O-aryl cleavage, while  $\text{C}_\alpha$ - $\text{C}_\beta$ -cleaved products accumulated for LE. Our research provides new insight on how fungi degrade lignin, which contributes to further optimizing the biological upgrading of lignocellulose.

**Keywords:** *Ceriporiopsis subvermispora*, *Lentinula edodes*, *Pleurotus eryngii*, selectivity, lignin degradation, lignin quantification, C<sub>α</sub>-oxidation, ligninolytic enzymes

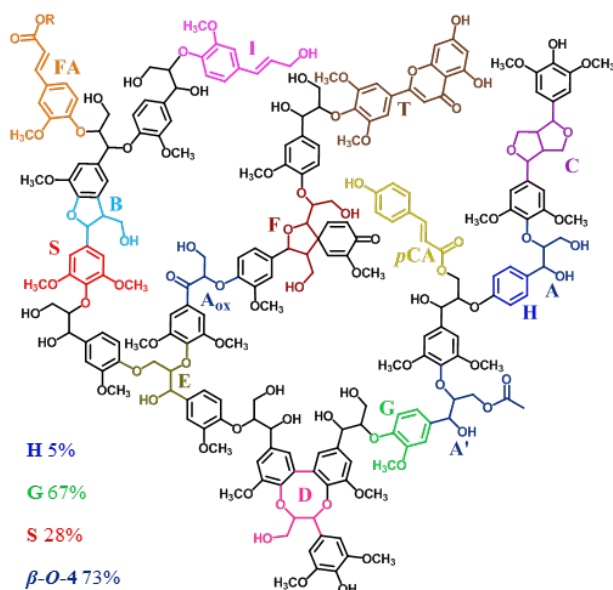
## INTRODUCTION

Lignocellulosic biomass, such as wheat straw or corn stover, is a valuable source of polysaccharides for the production of animal feed or biofuels and biochemical (Buranov and Mazza, 2008; Isroi *et al.*, 2011; Isikgor and Becer, 2015). The presence of lignin, however, hinders the conversion of these polysaccharides and, therefore, necessitates the use of pre-treatments aiming at lignin removal or degradation. These physical and/or chemical hydrothermal pre-treatments require extensive amounts of energy and chemicals (Behera *et al.*, 2014). A sustainable alternative is the use of white-rot fungi as biological pre-treatment and is increasingly receiving attention (Keller *et al.*, 2003; Isroi *et al.*, 2011; Tian *et al.*, 2012; Van Kuijk *et al.*, 2015a; Sindhu *et al.*, 2016). Among these fungi, the species *Ceriporiopsis subvermispora* (CS), *Pleurotus eryngii* (PE) and *Lentinula edodes* (LE) are particularly promising as they selectively removed lignin over cell wall polysaccharides. This results in a greatly enhanced enzymatic degradability of polysaccharides in further downstream processes (Van Kuijk *et al.*, 2015b; Van Kuijk *et al.*, 2017; Nayan *et al.*, 2018). To optimize the pretreatment with these fungi, mainly regarding selectivity and time-efficiency, it is important to understand delignification and the underlying mechanisms at molecular level. Such mechanistic insight facilitates the pinpointing of potential bottlenecks in the (enzymatic) process and might provide means to circumvent them, e.g. via supplementation of co-factors for the respective enzymes. Besides enabling control of the pre-treatment process, mechanistic insight expands our understanding of how fungi function in nature and how they contribute to terrestrial carbon-recycling.

Lignin is a heterogeneous phenolic polymer that, in grasses, is composed of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits, which are linked through a variety of carbon-carbon and aryl-ether linkages, with the  $\beta$ -O-4 ether as most abundant inter-unit linkage (~80%) (Ralph *et al.*, 2009a; Vanholme *et al.*, 2010; Del Río *et al.*, 2012). Structural complexity of grass-type lignin is further enhanced by the incorporation of *p*-

coumaric acid, ferulic acid and triclin in the macromolecule (Ralph 2010; Lan *et al.*, 2015; Li *et al.*, 2016). A schematic structure of wheat straw lignin is presented in Fig. 6.1.

Lignin degradation by white-rot fungi in general relies on a complex enzymatic machinery which is, depending on the species, mainly based on lignin peroxidases (LiP), manganese peroxidases (MnP), laccases (Lac) and the H<sub>2</sub>O<sub>2</sub> generating aryl-alcohol oxidases (AAO). Collectively, the catalytic performance of these enzymes results in the generation of radicals, i.e. aromatic or hydroxyl radicals, which can lead to various reactions such as C<sub>α</sub>-C<sub>β</sub> cleavage, breakdown of the β-O-4 ether, aromatic ring cleavage



**Fig. 6.1** Wheat straw lignin model including the most abundant substructures attempting to fairly represent the relative abundances of each moiety, based on literature (Ralph *et al.*, 2009a; Ralph 2010; Vanholme *et al.*, 2010; Del Río *et al.*, 2012; Lan *et al.*, 2015; Li *et al.*, 2016). Values in the figure are calculated from the structure drawn (excluding pCA, FA and T). H: *p*-hydroxyphenyl unit; G: guaiacyl unit; S: syringyl unit; A: β-O-4'alkyl-aryl ether; A': β-O-4'alkyl-aryl ether γ-acylated; A<sub>ox</sub>: β-O-4'alkyl-aryl ether C<sub>α</sub>-oxidized; B: phenylcoumaran; C: resinol; D: dibenzodioxocin; E: α,β-diarylether; F: spirodienone; I: cinnamyl alcohol (or aldehyde); T: triclin; pCA: *p*-coumarate; FA: ferulate (R = H or arabinose). Note that γ-acylation here is only shown on β-O-4 linkages, while in fact any free γ-OH can be acylated. For simplicity only monomeric FA is shown, though many diferulate linkages are known to exist. For more detailed structures see Fig. 6.5.



and demethoxylation, but also to polymerization (Ten Have and Teunissen 2001; Martínez *et al.*, 2005; Hatakka and Hammel 2011; Hernández-Ortega *et al.*, 2012; Camarero *et al.*, 2014).

Although CS, PE and LE all produce MnP, Lac and AAO, they differ in the number of genes encoding for these enzymes, which suggests different dependencies on the particular enzymes (Nagai *et al.*, 2007; Fernandez-Fueyo *et al.*, 2012; Yang *et al.*, 2016; Park *et al.*, 2017; Sakamoto *et al.*, 2017). Interestingly, none of these fungi possess genes encoding for LiP. Instead, versatile peroxidases were detected in PE, whereas CS was shown to produce two enzymes with lignin peroxidase and versatile peroxidase like activity (Fernández-Fueyo *et al.*, 2012; Yang *et al.*, 2016). It is clear from this variation in ligninolytic enzyme encoding genes that these fungi employ different strategies for delignification. However, whether these different strategies emerge into different underlying mechanisms remains unknown. Furthermore, it is poorly understood whether certain structural motifs of lignin are preferentially degraded or modified by the respective enzymatic machineries and, thus, lead to different residual lignin structures (Martínez *et al.*, 2005; Hatakka and Hammel 2011).

To the best of our knowledge, research on the conversion of grass lignin is limited (Camarero *et al.*, 1994; Crestini *et al.*, 1998; Martínez *et al.*, 2001), and studies where the fungi were compared under the same experimental conditions are scarce (Dorado *et al.*, 1999; Van Kuijk *et al.*, 2015b; Van Kuijk *et al.*, 2017). Furthermore, lignin degradation was, content-wise, only determined by using unspecific gravimetric analysis, which is thought to be particularly inaccurate for fungal-grown samples due to the presence of residual chitin and includes recalcitrant lignin only (Martínez *et al.*, 2005; Jurak *et al.*, 2015). Moreover, the scarce studies aiming at elucidating structural features of fungal-treated lignin *in situ* focused on using qualitative pyrolysis gas chromatography mass spectrometry (Py-GC/MS). Though considerable structural changes in fungal-treated wheat straw lignin could be revealed, mainly in terms of preference for phenolic lignin substructures and accumulation of C<sub>α</sub>-oxidized substructures, underlying mechanisms could not be elucidated (Camarero *et al.*, 1994; Van Kuijk *et al.*, 2015b; Van Kuijk *et al.*, 2017). More accurate and in-depth analysis of both lignin content as well as lignin structural features in fungal-treated biomass is expected to further resolve the employed lignin degrading mechanisms.

The recent development of a highly accurate pyrolysis gas chromatography mass spectrometry (Py-GC/MS) method that uses <sup>13</sup>C lignin as internal standard (<sup>13</sup>C-IS) offers a great opportunity to specifically quantify residual lignin content *in situ*, while

simultaneously providing structural insight (Van Erven *et al.*, 2017). In addition, developments in the use of heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) allow the analysis of whole cell wall samples *in situ*, without prior isolation or derivatisation of lignin (Kim *et al.*, 2008; Mansfield *et al.*, 2012). *In situ* analysis avoids the need for laborious lignin isolation procedures that may lead to partial extraction and polymer modification, especially for fungal-treated lignin (Björkman 1956; Kirk and Chang 1974; Chang *et al.*, 1975).

In this research, the combination of quantitative <sup>13</sup>C-IS Py-GC/MS and whole cell wall HSQC NMR was applied to fungal-treated wheat straw after growth of two strains of CS, PE and LE to obtain a better understanding of their delignification mechanisms. We found that both CS and LE outperform PE in terms of delignification effectivity and selectivity and, furthermore, vastly differed in residual lignin structures. We were able to identify that these lignin structures originated from fundamentally different delignification mechanisms, which is discussed in genomic context.

## MATERIALS AND METHODS

### Materials

All chemicals were obtained from commercial suppliers and used without further purification. Water used in all experiments was purified via a Milli-Q water system (Millipore, Billerica, MA, USA).

### Preparation of the fungal-treated wheat straw

Samples used in the present study were collected from a main experiment on selecting the best performing fungal strains based on their capability to improve the *in vitro* ruminal degradability of the treated straw (Nayan *et al.*, 2018). Two high potential strains from three different fungal species were selected: CS1 (CBS 347.63) and CS12 (ME-485) strains for *Ceriporiopsis subvermispora*, PE3 (Mycelia2600) and PE6 (AL04) for *Pleurotus eryngii* and LE8 (sh 03/08) and LE10 (LE75) for *Lentinula edodes*. Procedures for fungal strains preparation and pretreatment of the wheat straw has been previously described in detail (Nayan *et al.*, 2017). Briefly, all strains were maintained on malt extract agar before a piece of that agar (1.5 × 2.0 cm) was used to prepare the spawn for each fungus. The spawn was prepared using sterilized sorghum grains and was incubated at 24°C for up to 5 weeks. Organic wheat straw (*Triticum aestivum* L.) was purchased from a local farmer in the Netherlands and chopped into pieces of

approximately 3 cm. The wheat straw was soaked in water for 3 days at room temperature and excess water was drained for 5 h. Each container (185×185×78 mm, Combiness, Nevele, Belgium) was adjusted to contain  $90.2 \pm 0.3$  g of dry matter with a moisture content of ~74% w/w. After autoclaving at 121°C for 1 h, the straw was inoculated with the prepared spawn at 10% of the dry weight. The wheat straw (treated and untreated with fungi) was incubated in triplicate under solid state fermentation at 24°C in a climate-controlled chamber (relative humidity ~75%) for 1, 3 and 7 weeks. All samples were freeze-dried and ground over a 1 mm sieve using a cross beater mill (100AN, Peppink, Olst, the Netherlands). Since biological triplicates showed <5% RSD in conventional compositional feed analysis, they were thoroughly mixed in equal dry matter amounts to one replicate and ball-milled in a MM200 mixer mill (Retsch, Haan, Germany) for further analyses (Nayan *et al.*, 2018). We previously showed that untreated straws were stable during incubation and showed minimal variation (Nayan *et al.*, 2017). Therefore, the untreated wheat straw without incubation was used as control sample in this study.

### Carbohydrate content and composition

Carbohydrate content and composition was determined in duplicate by a modified method, using inositol as internal standard (Englyst and Cummings 1984). Samples were treated with 72% (w/w) H<sub>2</sub>SO<sub>4</sub> for 1 h at 30 °C followed by 1 M H<sub>2</sub>SO<sub>4</sub> for 3 h at 100 °C. The constituent monosaccharides released were analyzed as their alditol-acetate derivatives by using gas chromatography (Thermo Scientific, Synnyvale, CA, USA) and represented as anhydromonosaccharides. The uronic acids released after acid the hydrolysis step were determined in duplicate as anhydrouronic acid content by an automated meta-hydroxydiphenyl assay with addition of sodium tetraborate using an auto-analyzer (Skalar Analytical BV, Breda, The Netherlands) (Blumenkrantz and Asboe-Hansen 1973). Glucuronic acid (Fluka AG, Busch, Switzerland) was used as a reference (0 – 100 µg ml<sup>-1</sup>). Total carbohydrate content was calculated as the sum of neutral anhydrocarbohydrates and anhydrouronic acids.

### Semi-quantitative Py-GC/MS

Pyrolysis was performed with an EGA/PY-3030D Multi-shot pyrolyzer (Frontier Laboratories, New Ulm, MN, USA) equipped with an AS-1020E Autoshot auto-sampler as previously described (Jurak *et al.*, 2015; Van Erven *et al.*, 2017). The pyrolyzer was coupled to GC-MS using a Trace GC equipped with a DB-1701 fused-silica capillary column (30 m × 0.25 mm i.d. 0.25 µm film thickness) coupled to a DSQ-II mass

spectrometer (both Thermo Scientific, Waltham, MA, USA). Samples were weighed using a XP6 excellence-plus microbalance (Mettler Toledo, Columbus, OH, USA). Pyrolysis of total biomass (~80 µg) was performed at 500 °C for 1 min with an interface temperature of 300 °C. Pyrolysis products were injected on the column via split/splitless injection (at 250 °C) with a splitratio of 1:133 and helium was used as carrier gas with constant flow at 1.5 ml·min<sup>-1</sup>. The GC oven was programmed from 70 °C (2 min) to 270 °C at 5 °C·min<sup>-1</sup> and held at 270 °C for 15 min. MS detection was used with EI at 70 eV, a source temperature of 250 °C, a scan range of *m/z* 50-550 and a scan rate of 4.0 scans s<sup>-1</sup>. Compounds were identified by comparing retention time and mass spectrum with standards, the NIST library and data published by Ralph and Hatfield (1991).

For semi-quantitative analysis, pyrograms were processed by Xcalibur 2.2 software. The two most abundant fragments per compounds were automatically integrated using ICIS peak integration with optimized settings per compound. A manual correction was only applied when irregular peak shapes led to erroneous peak integration with method settings. Areas were normalized by dividing by corresponding relative response factors, as previously published, multiplied with the molecular weight of the respective compound and summed (Van Erven *et al.*, 2017). Lignin content was estimated on the basis of total area of lignin-derived pyrolysis products and compared to a wheat straw reference sample with known Klason lignin content (acid-insoluble lignin + acid-soluble lignin = 20.5 % w/w), as described by Jurak *et al.* (2015). Relative abundances of lignin-derived pyrolysis products were based on areas without molecular weight correction as previously described by Del Río *et al.* (2012), as RRF values are mole-based (Del Río *et al.*, 2012). Compounds were classified according to their structural features (Supporting Information Table 6-S1) and summed. All samples were prepared and analyzed in triplicate.

### **Quantitative Py-GC/MS with <sup>13</sup>C lignin as internal standard**

Pyrolysis was carried out as previously described in detail and in the section semi-quantitative Py-GC/MS (Van Erven *et al.*, 2017). Briefly, 10 µl of a <sup>13</sup>C lignin internal standard (IS) solution (1 mg ml<sup>-1</sup> ethanol/chloroform 50:50 v/v) was mixed with ~80 µg of sample and dried before analysis. Lignin-derived compounds of which fungal action increased the content above detection limits were identified by qualitative Py-GC/MS using full-MS detection and added to the existing SIM method. Lignin-derived pyrolysis products were monitored in selected ion monitoring (SIM) mode on the two most abundant fragments per compound (both <sup>12</sup>C and <sup>13</sup>C). The compound vanilloyl

acetaldehyde (VAL) was not properly detected in SIM due to a shifted segment and therefore was estimated from full MS analysis. The relative area of VAL versus vanillin as measured by full MS was thereto multiplied by the area of vanillin as measured by SIM. For correction of matrix-effects similar behavior as syringoyl acetaldehyde (SAL) was assumed. Areas for each compound were normalized by dividing by the respective relative response factor (RRF). RRF values were updated to system performance by recalculation to obtain an identical relative abundance of lignin-derived pyrolysis products of the  $^{13}\text{C}$  IS added to a wheat straw reference sample. Lignin content (% w/w) was determined on the sum of lignin-derived pyrolysis products, where RRF corrected areas for each compound were multiplied with the molecular weight of the respective compound and summed instead of the application of the published correction factor of 1.057 (Van Erven *et al.*, 2017). Areas were not corrected for molecular weight before relative abundance determination as previously described by Del Río *et al.* (2012), to allow direct comparison with data obtained by 2D-NMR analysis. Relative abundances of lignin-derived pyrolysis products were normalized for the  $^{13}\text{C}$  analogues from the IS present in the same sample to distinguish matrix and treatment effects. Compounds were classified according to their structural feature (Supporting Information Table 6-S1) and summed. All samples were prepared and analyzed in triplicate. Extractive-free samples were identical to total biomass samples for CS1 and therefore not measured for the other fungi.

### Whole cell wall 2D HSQC NMR spectroscopy

For NMR analysis of the whole cell wall material, ground wheat straw (1 mm) was successively extracted with acetone until the solvent became clear; followed by hot water treatment (100°C) for 3 h. The extractive-free samples were then freeze-dried before mixing biological replicates and fine milling in a PM100 planetary ball mill (Retsch, Haan, Germany). Two gram of sample was milled in a 50 ml  $\text{ZrO}_2$  beaker using 17  $\Phi$  10 mm balls of the same material at a frequency of 600 rpm with a net milling time of 4 h. After every 15 min of milling a pause of 10 min was set to avoid overheating of the sample. Around 100 mg of ball-milled sample was swollen in 0.75 ml DMSO- $d_6$ . 2D heteronuclear single quantum coherence (HSQC) NMR was performed according to previously described methods (Kim *et al.*, 2008; Del Río *et al.*, 2012; Mansfield *et al.*, 2012). The spectra were recorded at 25°C with Bruker's standard pulse sequence "hsqcetgpsisp2.2" on a Bruker AVANCE III 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm cryo-probe located at MAGNEFY (Magnetic resonance facility, Wageningen, The Netherlands). The spectral widths were 7,200 and 30,000 Hz

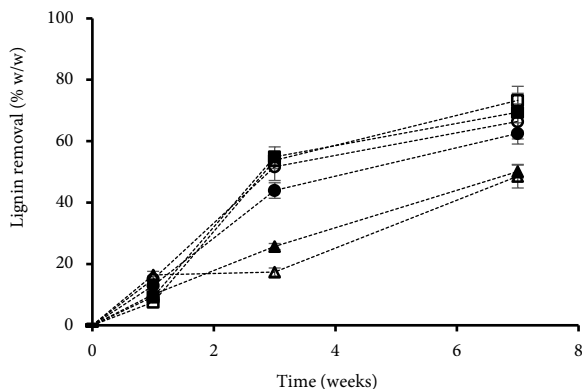
for the  $^1\text{H}$  and  $^{13}\text{C}$  dimensions, respectively. The number of collected complex points was 2018 in the  $^1\text{H}$  dimension with a relaxation time of 1 s. The number of collected scans was 16 and 400 increments of time were recorded in the  $^{13}\text{C}$  dimension. The  $^1J_{\text{CH}}$  used was 145 Hz.

The data was processed with Bruker TopSpin 3.5 software. Processing used were Gaussian apodization in  $^1\text{H}$  and a squared cosine function in  $^{13}\text{C}$  dimension. For  $^{13}\text{C}$ , data were zero filled up to 1024 points prior to Fourier transformation. The solvent peak (DMSO- $d_6$ ) was used as an internal reference ( $\delta_{\text{C}}$  39.5 ppm;  $\delta_{\text{H}}$  2.49 ppm). HSQC correlation peaks were assigned by comparison with literature (Ralph *et al.*, 2009b; Villaverde *et al.*, 2009; Rencoret *et al.*, 2010; Yuan *et al.*, 2011; Del Río *et al.*, 2012; Del Río *et al.*, 2015; Rencoret *et al.*, 2017; Das *et al.*, 2018). Semi-quantitative analysis of the volume integrals was performed according to Del Río *et al.* (2012) (Del Río *et al.*, 2012). Alternatively, in the aliphatic oxygenated region  $\beta$ -O-4 substructures were estimated from their  $\text{C}_{\beta}$ - $\text{H}_{\beta}$  correlations, since they were shown to be interfered to a lesser extent by the presence of carbohydrates (Mansfield *et al.*, 2012). For phenylcoumaran and resinol substructures their respective  $\text{C}_{\alpha}$ - $\text{H}_{\alpha}$  correlations were used. Volume integrals for resinol substructures were logically halved.  $\text{S}_{2,6}$ ,  $\text{G}_2$  and  $\text{H}_{2,6}$  signals were used for S, G and H units, respectively, where S and H integrals were halved as well. Oxidized analogues were estimated in a similar manner. Tricin, *p*CA and FA were similarly estimated from their respective  $\text{T}_{2,6}$ , *p*CA $_{2,6}$  and FA $_2$  signals. Volume integration was performed at equal contour levels. Amounts were calculated both as a percentage of total lignin ( $\text{H} + \text{G} + \text{G}_{\text{ox}} + \text{G}_{\text{mod}} + \text{S} + \text{S}_{\text{ox}} + \text{S}_{\text{mod}}$ ) and total lignin including *p*CA, FA and T (Sette *et al.*, 2011; Wen *et al.*, 2013).

## RESULTS AND DISCUSSION

### Wheat straw delignification during fungal growth

The extent of wheat straw delignification during growth of the different fungal strains was monitored by semi-quantitative Py-GC/MS (Fig. 6.2). This technique is not interfered by fungal cell wall material and the entire lignin population is measured (Martínez *et al.*, 2005; Jurak *et al.*, 2015). In comparison to conventional gravimetric analysis, semi-quantitative Py-GC/MS, therefore, is considered to provide more accurate insight in the delignification characteristics of the studied fungi. Clear lignin removal was observed



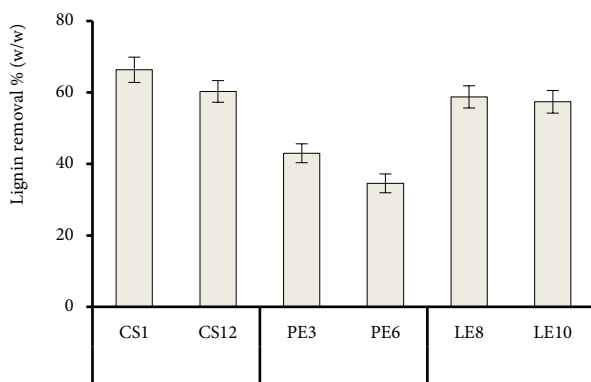
**Fig. 6.2.** Semi-quantitative Py-GC/MS determination of lignin removal from wheat straw during growth (1, 3 and 7 weeks) of *C. subvermispora* (CS), *P. eryngii* (PE) and *L. edodes* (LE) strains. □ CS1, ■ CS12, △ PE3, ▲ PE6, ○ LE8, ● LE10. Average and standard deviation of triplicates.

from the first week of growth and all strains extensively delignified the wheat straw within 7 weeks. CS and LE were the most effective species, which is in line with the initial evaluation of these fungi by conventional compositional feed analysis (Nayan *et al.*, 2018). Not only were CS and LE more effective, compared to PE, they also showed a distinct pattern of lignin removal in time. For CS and LE the major part (>70%) of the final lignin removal occurred within the first 3 weeks, whereas both PE strains showed a more gradual lignin removal in time, reaching less than 50% of the final lignin removal within 3 weeks. No significant differences were found in the extent of lignin removal after 7 weeks of growth between strains of the same species.

Though semi-quantitative Py-GC/MS clearly proved useful for the comparison of the delignification efficiency of the different fungi, we thrived for more accurate and precise quantification and characterization of the residual lignin. At the end-point of the treatment (7 weeks) the wheat straw was most affected by fungal growth and, for that reason, considered the most informative to further investigate the underlying delignification mechanisms (Van Kuijk *et al.*, 2016b; Van Kuijk *et al.*, 2017). Therefore, lignin in fungal-treated wheat straw after 7 weeks of growth was quantified by the recently developed quantitative  $^{13}\text{C}$ -IS Py-GC/MS method (Van Erven *et al.*, 2017). In this method, a  $^{13}\text{C}$  lignin internal standard is incorporated to correct for matrix effects and system performance, which was previously shown to improve the accuracy of lignin quantification in sound biomass samples (Van Erven *et al.*, 2017). From the total dry

matter recovered and lignin contents in the treated material, the amount of lignin removed was calculated and is presented in Fig. 6.3.

Although semi-quantitative Py-GC/MS gave fair estimates of lignin removal (Fig. 6.2), the method overestimated lignin removal in all samples by approximately 15%, mainly due to overestimation of the lignin content of the starting material (Supporting Information Table 6-S2). The results of quantitative Py-GC/MS confirmed the effectiveness of CS, LE and PE in degrading lignin, where in the case of CS and LE more than 60 %w/w of lignin was removed from the wheat straw within 7 weeks of treatment. The three fungal species, furthermore, were capable of removing lignin highly selectively over cell wall carbohydrates (Fig. 6-S1 and 6-S2). After 7 weeks of growth PE strains were found to have degraded significant amounts of glucan (8% w/w of initial glucan), whereas CS and LE left glucan less affected. Instead, these species degraded glucuron-arabinoxylan (GAX), but to a lesser extent than lignin. CS1 showed a remarkably higher degradation of GAX than CS12 (21 vs 5% w/w of initial GAX), while both LE strains degraded approximately 12% w/w of initial GAX. The selective removal of lignin over carbohydrates has been described in previous studies for the three fungi used. Though, more extensive hemicellulose degradation has been reported (Nayan *et al.*, 2017; Van Kuijk *et al.*, 2017; Nayan *et al.*, 2018). This is due to the gravimetric method that was used, in which solubilization and removal cannot be discriminated.



**Fig. 6.3** Quantitative  $^{13}\text{C}$ -IS Py-GC/MS determination of lignin degradation in wheat straw after 7 weeks of fungal growth. CS *Ceriporiopsis subvermispota*, PE *Pleurotus eryngii*, LE *Lentinula edodes*. Average and standard deviation of triplicates.



Based on absolute amounts of remaining polymers, the selectivity of lignin removal over glucan and GAX was the highest for CS, followed by LE and PE. In addition to the effectiveness of lignin removal, CS and LE, thus, also seem to outperform PE in terms of selectivity.

### Structural features of fungal treated lignin

Besides accurate lignin content, quantitative  $^{13}\text{C}$ -IS Py-GC/MS simultaneously provided accurate insight in the structural features of lignin (Van Erven *et al.*, 2017). The use of a  $^{13}\text{C}$ -IS lignin ensured that the observed changes in the residual lignin could indeed be ascribed to fungal action. The same samples were also subjected to 2D-HSQC NMR analysis allowing comparison with the  $^{13}\text{C}$ -IS Py-GC/MS data, which is discussed further on. Table 6.1 shows the relative abundances of the structural features of lignin of untreated and treated wheat straw. The bases of structural classification and relative abundances of individual lignin-derived pyrolysis products can be found in Supplementary Information Table 6-S1. The structural features of the untreated straw matched well with that of wheat straw that was previously analyzed in a similar manner, which confirmed that a representative wheat straw sample was used in our study (Van Erven *et al.*, 2017).

Unexpectedly, fungal treatment was found to minimally affect the overall composition of lignin subunits, with the largest effect observed for LE8 (H:G:S = 11:64:25) as compared to control (H:G:S = 10:62:28), which indicated that all lignin units were targeted in the process of lignin degradation. Although minimal, all fungi except CS12, showed a preference towards the removal of S units (over H- and G-units), as demonstrated by slightly lowered S/G ratios (e.g. 0.45 to 0.38 by LE8; Table 6.1). This preference was less pronounced compared to a previous study, where a factor 2 decrease in S/G ratio was found within 8 weeks of treatment by both CS and LE (Van Kuijk *et al.*, 2016b; Van Kuijk *et al.*, 2017). The main difference is the higher S/G ratio of the wheat straw used in the previous study (S/G = 0.7), compared to our wheat straw (S/G = 0.45). Hence, the most likely explanation of the lower S/G preference in our study was the difference in wheat straw used, which differed not only in the overall subunit composition, but might also have differed in the way these subunits were linked.

Preferential degradation of (sub-)structures with a higher degree of methoxylation has been related to: (1) a lower redox potential and (2) a reduced frequency of carbon-

**Table 6.1.** <sup>13</sup>C-IS Py-GC/MS relative abundance of control and fungal-spent wheat straw after 7 weeks of growth of *C. subvermispora* (CS), *P. eryngii* (PE) and *L. edodes* (LE) strains corrected for RRF and relative abundance of <sup>13</sup>C analogues. Average and standard deviation of triplicates.

	Control	CSI	CS12	PE3	PE6	LE8	LE10
Aromatic units (%)							
H	9.6 ± 0.4	12.0 ± 0.7	12.1 ± 0.3	10.0 ± 0.7	10.1 ± 0.8	11.1 ± 0.5	11.3 ± 0.5
G	62.2 ± 0.7	62.1 ± 0.5	59.3 ± 0.3	62.8 ± 1.4	63.3 ± 0.3	64.2 ± 0.2	63.0 ± 0.6
S	28.2 ± 0.5	26.0 ± 0.4	28.6 ± 0.1	27.2 ± 0.8	26.7 ± 0.9	24.7 ± 0.4	25.6 ± 0.1
S/G	0.45 ± 0.01	0.42 ± 0.01	0.48 ± 0.00	0.43 ± 0.02	0.42 ± 0.02	0.38 ± 0.01	0.41 ± 0.01
Structural moieties (%)							
Unsubstituted	4.6 ± 0.2	9.4 ± 0.5	9.4 ± 0.4	6.1 ± 0.4	5.8 ± 0.02	7.1 ± 0.4	7.7 ± 0.4
Methyl	2.0 ± 0.04	3.2 ± 0.3	3.4 ± 0.2	2.6 ± 0.1	2.5 ± 0.2	2.9 ± 0.1	2.9 ± 0.1
Vinyl	30.6 ± 1.0	30.1 ± 1.7	30.3 ± 0.3	29.6 ± 0.1	31.6 ± 1.2	31.7 ± 0.6	31.3 ± 0.7
4-vinylphenol	7.8 ± 0.4	7.7 ± 0.5	7.8 ± 0.3	7.3 ± 0.4	7.8 ± 0.6	7.8 ± 0.3	7.9 ± 0.4
4-vinylguaiacol	20.3 ± 0.7	20.4 ± 1.1	20.3 ± 0.04	19.9 ± 0.6	21.4 ± 0.6	21.7 ± 0.4	21.0 ± 0.7
C <sub>α</sub> -oxidized	3.7 ± 0.1	13.6 ± 0.6	14.7 ± 0.7	6.0 ± 0.3	5.2 ± 0.3	6.6 ± 0.1	6.9 ± 0.3
C <sub>α</sub> -oxidized G	2.0 ± 0.1	8.0 ± 0.3	8.1 ± 0.4	3.2 ± 0.2	2.7 ± 0.2	3.9 ± 0.1	4.0 ± 0.2
C <sub>α</sub> -oxidized S	1.6 ± 0.04	5.6 ± 0.3	6.6 ± 0.3	2.7 ± 0.2	2.5 ± 0.1	2.7 ± 0.03	3.0 ± 0.1
Acetaldehyde	0.4 ± 0.02	7.7 ± 0.4	7.9 ± 0.5	1.9 ± 0.2	1.9 ± 0.2	2.7 ± 0.1	2.9 ± 0.2
C <sub>β</sub> -ox	1.4 ± 0.02	2.5 ± 0.1	2.7 ± 0.1	1.7 ± 0.1	1.6 ± 0.05	1.8 ± 0.03	1.8 ± 0.04
C <sub>γ</sub> -ox	54.8 ± 0.9	45.3 ± 2.3	43.9 ± 1.4	52.1 ± 0.6	51.4 ± 1.4	48.8 ± 0.6	48.6 ± 0.6
Miscellaneous	3.3 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.9 ± 0.1	3.5 ± 0.5	3.8 ± 0.1	3.8 ± 0.2
PhC <sub>γ</sub> <sup>a</sup>	58.5 ± 1.0	50.0 ± 2.3	48.7 ± 0.8	56.6 ± 0.6	55.4 ± 1.6	53.3 ± 0.6	53.0 ± 0.5
PhC <sub>γ</sub> -Acetaldehyde <sup>b</sup>	58.1 ± 0.9	42.4 ± 2.3	40.8 ± 1.3	54.7 ± 0.8	53.9 ± 1.7	50.6 ± 0.7	50.2 ± 0.3

<sup>a</sup> phenols with intact  $\alpha,\beta,\gamma$  carbon side chain.

<sup>b</sup> phenols with intact  $\alpha,\beta,\gamma$  carbon side chain, without acetaldehydes.

carbon ('condensed') substructures (Martínez *et al.*, 2001; Del Río *et al.*, 2002; van Kuijk *et al.*, 2016b; Van Kuijk *et al.*, 2017). Still, as mentioned above, CS, PE and LE did not show a clear preference for degrading the more methoxylated S-units over G- and H-units in our research. Demethoxylation, due to which S units 'transform' into G units, and likewise G units into H units, is known to occur due to fungal action, and might, however, potentially have masked the preferential removal of certain subunits (Martínez *et al.*, 2005; Camarero *et al.*, 2014).

When categorized according to structural moiety, clear effects of fungal growth on residual lignin structures were observed (Table 6.1). Most pronounced was the increase of unsubstituted and C $\alpha$ -oxidized pyrolysis products at the expense of products with three carbons in the side-chain (PhC $\gamma$ ) in fungal-treated straw. The relative abundances of these moieties greatly varied across species, with the CS strains showing the highest increases in unsubstituted (two-fold) and C $\alpha$ -oxidized (five-fold) substructures as compared to untreated wheat straw. Again, minimal differences were found between strains of the same species. These observations are in line with previous Py-GC/MS studies on fungal-treated biomass and have been suggested to indicate degradation of inter-unit linkages within the lignin macromolecule (Del Río *et al.*, 2002; Choi *et al.*, 2006; Van Kuijk *et al.*, 2016b; Van Kuijk *et al.*, 2017). To add to these previous suggestions, we hypothesize that in particular the relative abundance of PhC $\gamma$  is a measure for the amount of intact inter-unit linkages present, which is substantiated by the NMR data shown below. To explain, it is well known that upon pyrolysis of lignin, the intact ( $\beta$ -O-4) inter-unit linkages, consisting of C $\alpha$ -C $\beta$ -C $\gamma$  aliphatic chains, decompose into products with various structures and chain-lengths (unsubstituted, C $\alpha$ , C $\beta$  and C $\gamma$  side-chains) (Ralph and Hatfield 1991). At the pyrolysis conditions applied in our study, secondary reactions are absent. Therefore, lignin pyrolysis mainly yields monomeric products that are a direct result of bond-cleavage (Patwardhan *et al.*, 2011). Hence, the maximum chain length of the pyrolysis products equals the chain length of the substructures they originate from. PhC $\gamma$  products can, thus, only form from structures in which the three carbon side-chain was initially present, being mainly intact inter-unit linkages. Further, since the content of pendant cinnamyl alcohol and cinnamyl aldehyde end-groups in wheat straw was found to be rather low (Del Río *et al.*, 2012; Zeng *et al.*, 2013), their contribution to PhC $\gamma$  products is considered marginal.

Interestingly, for both *C. subvermisporea* strains, the increase in C $\alpha$ -oxidized pyrolysis products was for more than half determined by the two compounds vanilloyl acetaldehyde (VAL) and syringoyl acetaldehyde (SAL). These compounds are respectively

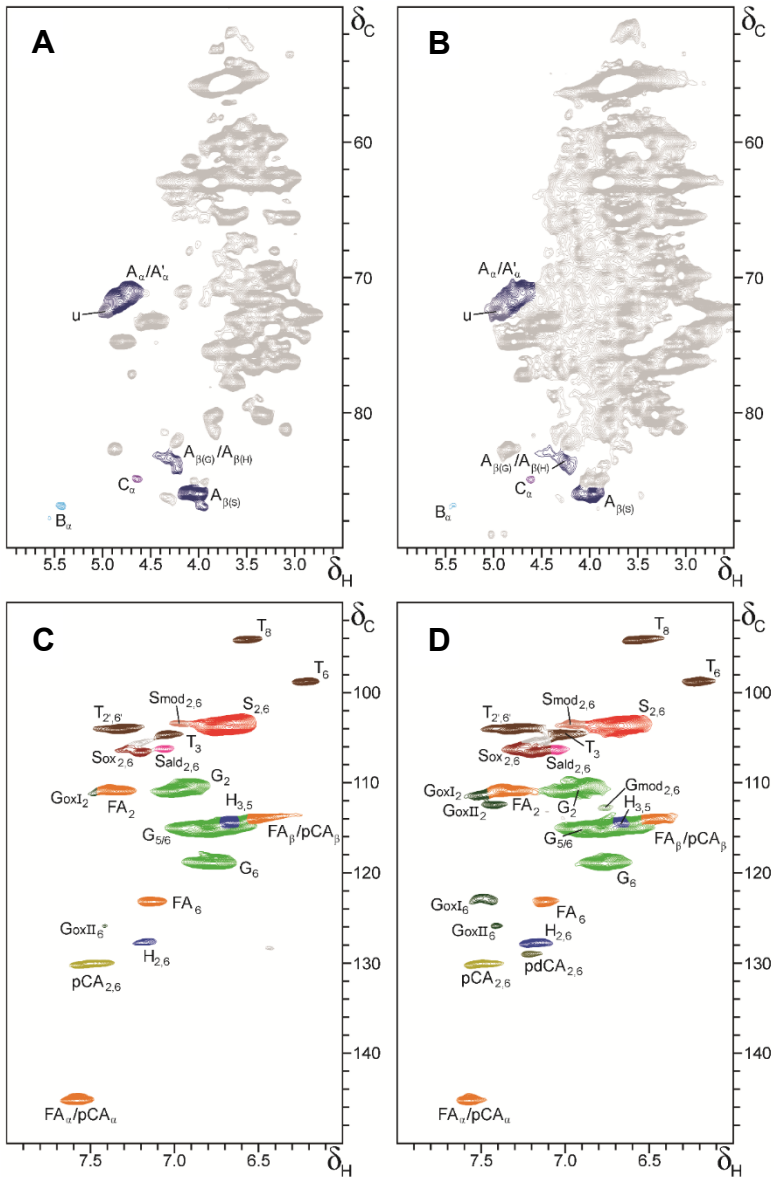
G and S-unit derivatives with three carbons in the side-chain and a ketone at the  $C_\alpha$  position (Supporting Information Table 6-S1, Fig. 6-S3). Analogous to the explanation given above for PhC $_\gamma$  products, we hypothesize that VAL and SAL solely formed from oxidized  $\beta$ -O-4 linkages and/or their cleavage products of which the three carbon ( $\alpha$ ,  $\beta$  and  $\gamma$ ) side chain remained intact. To challenge this hypothesis, it can be postulated that pyrolysis of triclin (Fig. 6.5) can result in SAL from the cleavage of the A-ring and rearrangement of the double bond. However, since we observed no SAL in the pyrograms of triclin-enriched extracts, triclin can be ruled out as potential (interfering) source of acetaldehyde products. If indeed acetaldehyde products originated from cleavage products of the inter-unit linkages, rather than oxidized  $\beta$ -O-4 linkages, they should be subtracted from the PhC $_\gamma$  for a more fair representation of the intact inter-unit linkages (PhC $_\gamma$ -acetaldehyde in Table 6.1). Our results, thus, suggest that fungal-treated lignin was considerably reduced in intact inter-unit linkages, in particular for CS strains (-30% compared to control). As  $C_\alpha$ -oxidized products greatly accumulated, this reduction in intact inter-unit linkages was likely caused by oxidative cleavage.

Semi-quantitative Py-GC/MS confirmed that the observed structures were a direct effect of lignin degradation, i.e. that they accumulated during fungal growth as lignin degradation proceeded (Supporting Information Fig. 6-S3). Intriguingly, though CS and LE followed a highly similar pattern of lignin degradation in time (Fig. 6.2), they vastly differed in the remaining lignin structures after their growth. To further substantiate these findings, we also assessed the residual lignin structures by whole cell wall 2D-NMR.

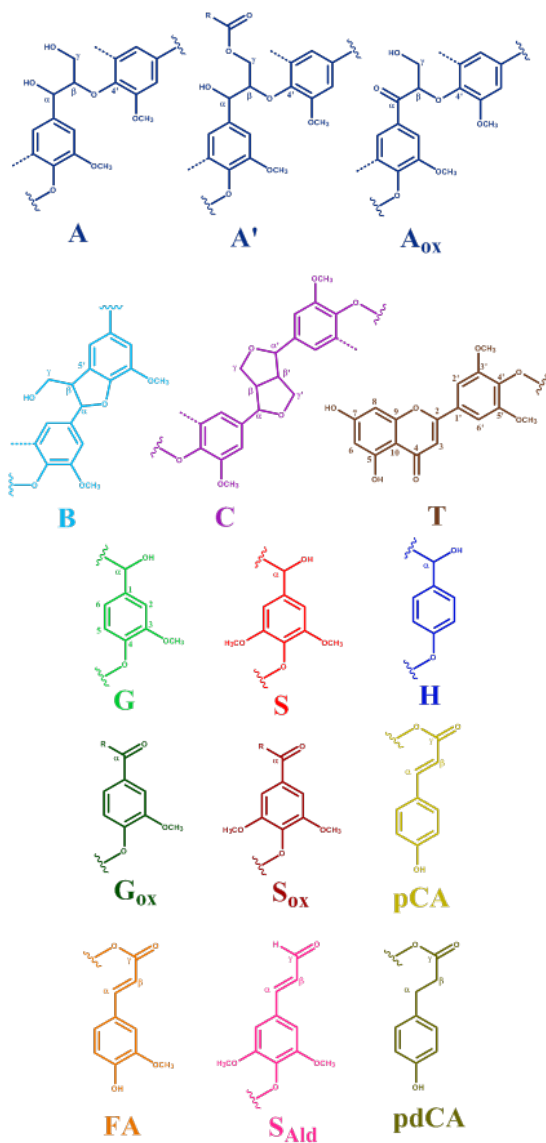
### *2D HSQC NMR of fungal-treated straw.*

Sample preparation for *in situ* whole cell wall gel state 2D-NMR requires finely divided, planetary ball milled samples. Planetary ball milling is associated with structural changes in lignin, mainly with regards to the cleavage of  $\beta$ -O-4 inter-unit linkages (Fujimoto *et al.*, 2005).  $^{13}\text{C}$ -IS Py-GC/MS analysis of the ball-milled samples showed that the milling did not induce structural changes in the lignin present and that the inter-unit linkages remained unaffected (PhC $_\gamma$ , constant). Thus, 2D NMR could be appropriately used.

The aliphatic ( $\delta_{\text{C}}/\delta_{\text{H}}$  50-90/2.5-6.0) and aromatic/unsaturated ( $\delta_{\text{C}}/\delta_{\text{H}}$  90-160/6.0-8.0) regions of the recorded HSQC spectra of untreated and CS1 treated wheat straw are presented in Fig. 6.4, with structures of the assigned correlation peaks shown in Fig. 6.5,



**Fig. 6.4:** HSQC NMR spectra of control (A, C) and CS1-treated wheat straw (B, D). A,B: aliphatic ( $\delta_C/\delta_H$  50-90/2.5-6.0) region, C,D: aromatic/unsaturated ( $\delta_C/\delta_H$  90-160/6.0-8.0) region. Structures of annotated correlation peaks are presented in Fig. 6.5.  $GoxII_2$  and  $GoxII_6$  are tentatively assigned.  $G_{\text{mod}}$  and  $S_{\text{mod}}$ , being unknown derivatives of G and S units, respectively, are presented in a lighter color of the original subunit. Carbohydrate and unassigned signals are presented in gray. u: unassigned signal overlapping  $A_\alpha/A'_\alpha$ .



**Fig. 6.5:** Structures annotated by HSQC NMR. **A:**  $\beta$ -*O*-4' alkyl-aryl ether; **A'**:  $\beta$ -*O*-4' alkyl-aryl ether  $\gamma$ -acylated; **A<sub>ox</sub>**:  $\beta$ -*O*-4' alkyl-aryl ether  $C_{\alpha}$ -oxidized; **B:** phenylcoumaran; **C:** resinol; **T:** tricrin; **pCA:** *p*-coumarate; **pdCA:** *p*-dihydrocoumarate; **FA:** ferulate; **G:** guaiacyl unit; **G<sub>ox</sub>**:  $C_{\alpha}$ -oxidized guaiacyl unit; **S:** syringyl unit; **S<sub>ox</sub>**:  $C_{\alpha}$ -oxidized syringyl unit; **H:** *p*-hydroxyphenyl unit; **S<sub>Ald</sub>**: syringaldehyde; Dotted line represents -H or -OCH<sub>3</sub>. R in G<sub>ox</sub> and S<sub>ox</sub> can be a side chain in ketones or hydroxyl in carboxylic acids. Wavy lines indicate main positions of further coupling. Colors match the assigned contours in Fig. 6.4.

of which chemical shift assignments were based on literature and shown in Supplementary Information Table 6-S3 (Ralph *et al.*, 2009b; Villaverde *et al.*, 2009; Rencoret *et al.*, 2010; Yuan *et al.*, 2011; Del Río *et al.*, 2012; Del Río *et al.*, 2015; Rencoret *et al.*, 2017; Das *et al.*, 2018). Aliphatic and aromatic/unsaturated regions of the HSQC spectra of the wheat straw treated by the other fungi are shown in Supporting Information Fig. 6-S3. The aliphatic regions were dominated by polysaccharide signals, mainly derived from xylan and acetylated xylan moieties, which (partially) overlapped with  $\beta$ -O-4' substructures. This overlap was more severe for fungal-treated samples, from which lignin was selectively removed. Despite this overlap, phenylcoumaran ( $\beta$ -5') and resinol ( $\beta$ - $\beta$ ) substructures were readily observed, whereas C $_{\alpha}$ -oxidized ( $\alpha$ -keto)  $\beta$ -O-4', dibenzodioxocins (5-5'/4-O- $\beta$ ), spirodienones ( $\beta$ -1'/ $\alpha$ -O- $\alpha'$ ) and  $\alpha,\beta$ -diaryl ether linkages were only observed at higher zoom levels. At these zoom levels other contours were not resolved anymore, which would result in a lower accuracy of volume integration.

In the aromatic region, clear lignin signals, typical for wheat straw, were observed (S<sub>2,6</sub>, G<sub>2</sub>, H<sub>2,6</sub> and several tricin (T), pCA and FA related signals) (Del Río *et al.*, 2012; Zeng *et al.*, 2013). Interestingly, in the fungal-treated samples C $_{\alpha}$ -oxidized aromatic units became more apparent, especially after treatment with CS and is discussed in detail below. Although C $_{\alpha}$ -oxidized S-units ( $\delta_C/\delta_H$  106.6/7.2) are commonly found in NMR studies on (native) lignin, C $_{\alpha}$ -oxidized G-units ( $\delta_C/\delta_H$  111.2/7.5) are less often described and have, hitherto, never been observed at the levels shown here (Yuan *et al.*, 2011; Rencoret *et al.*, 2016; Rencoret *et al.*, 2017). Notably, two different C $_{\alpha}$ -oxidized G-units substructures could be distinguished, although their exact structures remained unidentified (GoxI<sub>2</sub>  $\delta_C/\delta_H$  111.2/7.5, GoxII<sub>2</sub>  $\delta_C/\delta_H$  112.4/7.4). Another structural aspect resulting from fungal action, is the increased intensity of derivatives of both S (S<sub>mod</sub>,  $\delta_C/\delta_H$  103.4/6.96) and G units (G<sub>mod</sub>,  $\delta_C/\delta_H$  112.8/6.76). Though the NMR database of lignin and cell wall model compounds could confirm that these signals originated from either S or G units, their exact structures could not be elucidated (Ralph *et al.*, 2009b). To identify both the unidentified oxidized G-units and S/G-derivatives, future extensive NMR-research is needed, not being the aim of the current research. A summary of the semi-quantitative analysis of the volume integrals of aromatic units and inter-unit linkages (see materials and methods for details) is given in Table 6.2.

Similar to <sup>13</sup>C-IS Py-GC/MS (Table 6.1), 2D-NMR showed minor changes in H:G:S composition after fungal treatment (Table 6.2). For instance, the H:G:S composition changed from 2:54:44 in control to 4:56:36 in CS1-treated straw. Again a (slight)

**Table 6.2.** Structural characteristics of lignin in wheat straw treated with different strains of *C. subvermispora* (CS), *P. eryngii* (PE) and *L. edodes* (LE) for 7 weeks, using semi-quantitative HSQC NMR analysis.

	Control	CS1	CS12	PE3	PE6	LE8	LE10
Lignin subunits (%) <sup>a</sup>							
H	2 (6)	4 (8)	4 (8)	3 (7)	2 (6)	3 (7)	4 (7)
G	52 (53)	46 (48)	42 (48)	56 (60)	53 (56)	53 (57)	57 (56)
G <sub>ox</sub>	2 (1)	10 (8)	12 (10)	2 (2)	3 (3)	6 (5)	4 (3)
G <sub>mod</sub>	0 (0)	3 (2)	4 (3)	0 (0)	1 (1)	0 (0)	0 (0)
S	37 (34)	24 (26)	25 (20)	31 (26)	32 (27)	30 (24)	29 (29)
S <sub>ox</sub>	5 (4)	9 (7)	12 (10)	6 (5)	6 (5)	6 (5)	5 (4)
S <sub>mod</sub>	2 (1)	3 (2)	2 (2)	1 (1)	1 (1)	2 (2)	1 (1)
S/G	0.81 (0.72)	0.62 (0.60)	0.67 (0.64)	0.65 (0.59)	0.69 (0.64)	0.63 (0.60)	0.58 (0.59)
Hydroxycinnamates (%) <sup>b</sup>							
<i>P</i> -coumarate	6 (5)	4 (4)	4 (4)	5 (4)	5 (4)	4 (4)	4 (4)
<i>P</i> -dihydrocoumarate	0 (0)	2 (1)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)
ferulate	21 (15)	19 (14)	17 (13)	15 (12)	15 (12)	16 (13)	17 (13)
Lignan (%) <sup>b</sup>							
tricin	10 (7)	11 (8)	9 (7)	6 (5)	7 (5)	8 (6)	10 (8)
Lignin inter-unit linkages (%) <sup>c</sup>							
$\beta$ -O-4' aryl ethers	43 (90)	36 (93)	31 (97)	39 (90)	39 (85)	40 (86)	39 (88)
phenylcoumarans	3 (6)	2 (4)	1 (2)	3 (7)	5 (11)	4 (10)	3 (7)
resinols	1 (3)	1 (3)	0 (1)	1 (3)	2 (4)	2 (4)	2 (4)
total	48 (100)	39 (100)	32 (100)	43 (100)	46 (100)	46 (100)	45 (100)

<sup>a</sup> percentage of subunits (H+G+G<sub>ox</sub>+G<sub>mod</sub>+S+S<sub>ox</sub>+S<sub>mod</sub>=100) and in parenthesis (subunits+pCA(H)+pCA(H)+FA(G)+T(S) = 100).<sup>b</sup> percentage versus aromatic units and subunits + pCA+pCA+FA+T (see subscript a).<sup>c</sup> percentage of inter-unit linkages versus total subunits and in parenthesis as percentage of total inter-unit linkages.



preferential removal of S-units was observed, thereby confirming our previous findings by Py-GC/MS. However, for a fair comparison of the H:G:S composition found by the two techniques, it should be considered that whereas the hydroxycinnamic acids (*p*CA and FA) and tricin (T) can be distinguished from ‘core-lignin’ substructures in 2D-NMR, in Py-GC/MS they result in similar pyrolysis products. Upon pyrolysis both *p*CA and FA as well as inter-unit linkages result in the formation of 4-vinylphenol and 4-vinylguaiacol, respectively. Analogous, tricin is expected to result in S-unit derivatives upon pyrolysis.

To enable a comparison of both techniques in terms of H:G:S composition (mol based), *p*CA (H), FA (G) and T (S) were included in the determination of the subunit composition by NMR (Table 6.2, numbers in parentheses). Besides analytical comparability, the obtained subunit composition is, to our opinion, more representative of ‘true lignin’ as these substructures were shown to be an integral part of lignin (Ralph 2010; Lan *et al.*, 2016). Upon inclusion of these substructures the obtained subunit compositions found by both techniques matched better, though they remained different (CS1 H:G:S (NMR) = 8:58:35, H:G:S (Py-GC/MS) = 12:62:26). It cannot be expected that both techniques result in identical values, as different entities are measured (Mansfield *et al.*, 2012). Still, because similar trends were observed, complementary structural information can be obtained by comparing both techniques. One of the explanations for the observed difference is that the semi-quantitative nature of the NMR technique results in over-estimation of end-units as a result of differences in relaxation behavior compared to ‘core-lignin’ (Mansfield *et al.*, 2012). Another explanation is that hydroxycinnamic acids are pyrolyzed more efficiently compared to ‘core-lignin’, which results in overestimation of their abundances and contributions to lignin (Del Río *et al.*, 2012).

Most pronounced changes based on NMR was the significant increase in  $C_{\alpha}$ -oxidized aromatic units in fungal-treated samples, with a three-fold accumulation in CS-treated lignin (19-24% of total aromatic units; Table 6.2). This increase coincides with the increase in  $C_{\alpha}$ -oxidized products in Py-GC/MS and these compounds, hence, were mostly derived from  $C_{\alpha}$ -oxidized units initially present in the polymer. Even, relative abundances (% mol) of  $C_{\alpha}$ -oxidized substructures obtained by both techniques were in fairly good accordance (CS1  $C_{\alpha}$ -ox (NMR) = 15%,  $C_{\alpha}$ -ox (Py-GC/MS) = 14%). It can be assumed that the  $C_{\alpha}$ -oxidized substructures accumulated in fungal-treated lignin due to the fact that, once oxidized, these units cannot be further oxidized (by the respective fungal oxidases present) and are therefore resistant against further degradation and further metabolization.  $C_{\alpha}$ -oxidized S-units appeared more prone to accumulate than their  $C_{\alpha}$ -oxidized G-unit counterparts ( $S_{ox}/S > G_{ox}/G$ ) for all fungi. This might on the one hand be

explained by their ease of formation, as discussed in the previous section. On the other hand, S-units might be more difficult to be consumed and fully metabolized.

Interestingly, while the C<sub>α</sub>-oxidized aromatic units clearly increased, no apparent increase of C<sub>α</sub>-oxidized ( $\alpha$ -keto)  $\beta$ -O-4' substructures could be observed. The latter observation shows that the C<sub>α</sub>-oxidized signals mainly originated from cleaved linkages, and simultaneously highlights the importance of corresponding VAL and SAL compounds in the Py-GC/MS analysis. The suggestion that cleaved linkages accumulated in the residual lignin is further substantiated by the reduced amounts of intact inter-unit linkages in fungal-treated lignin. Intact inter-unit linkages were severely reduced by CS treatment (CS1 -20%, CS12 -34%; Table 6.2), which was also observed by Py-GC/MS. The abundance of PhC<sub>7</sub> products excluding acetaldehydes, thus, seems to be a highly valuable parameter for the investigation of lignin structure by Py-GC/MS.

Previous studies also reported on the reduction of inter-unit linkages in CS-treated lignin (Guerra *et al.*, 2004; Choi *et al.*, 2006). However, these studies were conducted on wood, of which the lignin is of different structure. Still, in these biomasses the  $\beta$ -O-4 inter-unit linkage is most abundant and, therefore, can be used to compare fungal action. Guerra *et al.* (2004) found that the CS-treated pine was preferentially reduced in  $\beta$ -O-4 linkages, while carbon-carbon linked units were more resistant against fungal degradation (Guerra *et al.*, 2004). Our results, on the contrary, indicated that all inter-unit linkages in wheat straw lignin were comparable in their susceptibility towards fungal degradation as their relative composition was rather stable (<10% different from control; Table 6.2). Another significant result from 2D-NMR was the reduction of FA moieties after fungal treatment, particularly by PE strains (around 30% reduction; Table 6.2). Possibly, the lignin-carbohydrate linkages in which FA participates, were targeted in a more preferential manner (Ralph 2010; Hatfield *et al.*, 2017). Though, the specific removal of glucuronoarabinoxylan (GAX) linked ferulates and crosslinked diferulates cannot be excluded. Such structures could be cleaved by for example feruloyl esterases, shown to occur extracellularly for PE and LE (Haase-Aschoff *et al.*, 2013).

The specific targeting of FA moieties is in line with the concurrent lignin and GAX degradation during fungal growth. The magnitudes of FA and GAX removal were, however, not directly linked (Fig. 6-S1 and 6-S2). In contrast to FA, *p*CA moieties were not preferentially removed, but shown to be partially reduced to dihydro analogues (Table 6.2). Esterified at the  $\gamma$ -OH, *p*CA moieties are in the vicinity of the bonds that are being oxidized, and, therefore, might play a role in redox cycling.

Tricin was surprisingly shown to be specifically targeted during fungal growth, in particular by PE strains (-40% vs total lignin; Table 6.2). Tricin has recently been suggested to play an important role in the biosynthesis of lignin in poaceous crops, by functioning as a nucleation site for lignin formation (Del Río *et al.*, 2012; Lan *et al.*, 2015; Lan *et al.*, 2016; Li *et al.*, 2016). The preferential removal of triclin suggests that, besides lignin formation, triclin may play a crucial role in the fungal degradation of lignin as well.

### **Mechanistic insight in fungal delignification by combining results of $^{13}\text{C}$ -IS Py-GC/MS and 2D-NMR**

Excitingly, the combination of 2D-NMR and  $^{13}\text{C}$ -IS Py-GC/MS gave more insight in the reaction mechanisms underlying delignification by the studied fungi, in particular for CS strains. The absence of  $\text{C}_\alpha$ -oxidized ( $\alpha$ -keto)  $\beta$ - $O$ -4' substructures in 2D-NMR combined with decreased amounts of intact inter-unit linkages (both techniques) confirmed that the  $\text{C}_\alpha$ -oxidized substructures were present in the fungal-treated lignin macromolecule as cleavage products of the inter-unit linkages. Upon pyrolysis, these cleavage products resulted in the formation of the acetaldehydes VAL and SAL. Since these pyrolysis products contain an intact  $\alpha$ ,  $\beta$ ,  $\gamma$  carbon side chain, the structures they originate from should contain this three-carbon side chain as well. Hence, we postulate that acetaldehyde pyrolysis products originated from remaining structures after  $\text{C}_\beta$ - $O$  cleavage of the  $\beta$ - $O$ -4' ether.  $\text{C}_\alpha$ -oxidized pyrolysis products with an  $\alpha$ -carbon side-chain, like vanillin and syringaldehyde, were likewise, but not specifically, formed from cleavage products and thus are suggested to be the result of  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage.

The high proportion of acetaldehyde pyrolysis products formed from CS-treated lignin suggests that  $\text{C}_\beta$ - $O$ -aryl cleavage was the main underlying mechanism of lignin degradation, while for PE and LE on the contrary  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage predominated. The latter two species, however, showed less accumulation of  $\text{C}_\alpha$ -oxidized substructures in treated lignin, at the respective levels of lignin removal. This might indicate that other cleavage reactions not yielding  $\text{C}_\alpha$ -oxidation might be more dominant than previously thought (Crestini *et al.*, 2003). On the other hand, if  $\text{C}_\beta$ - $O$ -aryl cleavage did occur, but the resulting intermediate products were further degraded in follow-up reactions, we cannot pick this up in residual lignin structures after 7 weeks of fungal growth. Collectively, the enzymes produced by LE and PE might thus be able to degrade each lignin subunit further, without resulting in increased, content-wise, lignin degradation. Our result clearly put forward that the delignification mechanism of CS is fundamentally different from LE and PE.

Though the genomes of the studied fungi are known to differ considerably, the number of genes encoding for ligninolytic enzymes seems inadequate to explain the mechanistic differences observed between the studied fungi, primarily because of the lack of transcriptomic data and insight into the activities of the enzymes once produced (Nagai *et al.*, 2007; Fernandez-Fueyo *et al.*, 2012; Fernández-Fueyo *et al.*, 2012; Yang *et al.*, 2016; Park *et al.*, 2017; Sakamoto *et al.*, 2017). Given the number of genes encoding for the ligninolytic enzymes of these fungi (Table 6.3), PE and LE seem to rely mainly on laccase, while a more prominent role of MnP is suggested for CS. This is supported by the fact that delignification of CS was inhibited when the  $Mn^{2+}$  concentration was severely reduced and stimulated by addition of  $Mn^{2+}$ , whereas  $Mn^{2+}$  addition did not stimulate delignification by LE (Jensen *et al.*, 1996; Van Kuijk *et al.*, 2016a). Moreover, it was shown that, in the presence of unsaturated fatty acids, MnP can degrade lignin model compounds via lipid peroxidation into products that, upon pyrolysis, could yield the acetaldehyde products we observed (Bao *et al.*, 1994; Jensen *et al.*, 1996). Interestingly, a similar MnP-peroxidation reaction was suggested for alkylitaconic acids, secondary metabolites that are produced by CS but not by LE and PE. As peroxidation was shown to shift the  $\beta$ -O-4' cleavage reaction more towards C $\beta$ -O-aryl cleavage, it can be postulated that the presence of alkylitaconic acids underlies the observed predominance of this cleavage reaction by CS. However, laccases and the recently identified enzymes with a LiP/VP like activity could have resulted in similar products, due to which unambiguity regarding the underlying enzyme system remains (Srebotnik *et al.*, 1997; Kawai *et al.*, 2002; Cantarella *et al.*, 2003; Yelle *et al.*, 2014). For further validation and

**Table 6.3.** Number of genes encoding for ligninolytic enzymes in *C. subvermispora* (CS), *P. eryngii* (PE) and *L. edodes* (LE). Based on Nagai *et al.* (2007), Fernandez-Fueyo *et al.* (2012), Yang *et al.* (2016), Park *et al.* (2017) and Sakamoto *et al.* (2017).

Enzyme	CS	PE	LE
LiP	N/D	N/D	N/D
MnP	13	4	3
VP	N/D	2	N/D
Laccase	7	9	11
AAO	5	10	4
LiP/VP like	2	N/D	N/D

LiP: lignin peroxidase, MnP: manganese peroxidase, VP: versatile peroxidase, AAO: aryl alcohol oxidase, N/D: not detected.

interpretation of our observations isolation of lignin from treated wheat straw during fungal growth combined with comprehensive structural analysis (e.g. quantitative combinatorial  $^{13}\text{C}/\text{HSQC}$  NMR) should be applied next to proteomics and transcriptomics.

## CONCLUSIONS

Our results show that *Ceriporiopsis subvermispota* (CS) and *Lentinula edodes* (LE) are more effective and selective wheat straw lignin degrading species than *Pleurotus eryngii* (PE). They degraded more than 60 % (w/w) of lignin without extensive carbohydrate degradation within 7 weeks of treatment. Though CS and LE followed a similar pattern of lignin degradation in time, the structural-features of residual lignin as determined by quantitative  $^{13}\text{C}$ -IS Py-GC/MS and *in situ* HSQC NMR were vastly different. For example, both techniques revealed that CS-treated lignin was exceptionally high in  $\text{C}_\alpha$ -oxidized substructures (up to 24% of aromatic units) and a factor two higher than LE-treated lignin. LE and PE, on the other hand, more specifically targeted ferulic acid substructures, while PE removed triclin up to 40% more preferentially than other substructures. Furthermore, CS delignification mainly proceeded via  $\text{C}_\beta$ -*O*-aryl and  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage of the lignin inter-unit linkages, while inter-unit degradation by LE and PE seemed dominated by  $\text{C}_\alpha$ - $\text{C}_\beta$  cleavage. We, therefore, suggest that the underlying delignification mechanisms of these fungi are fundamentally different. Besides assisting the further optimization of fungal pre-treatment of plant biomass we highlight that the choice of fungus impacts the structure of residual lignin and results in lignin with remarkable structure. Thus, fungal pretreatment not only enhances the degradability of plant cell wall polysaccharides, but also results in an interesting lignin fraction that can be exploited to further increase the sustainability of the process.

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Supplementary information for:

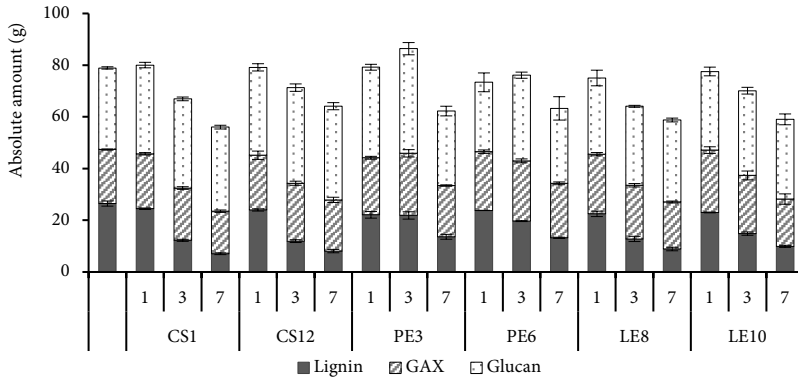
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**Mechanistic insight in the selective delignification of wheat straw by three white-rot fungal species through quantitative  $^{13}\text{C}$ -IS Py-GC/MS and whole cell wall HSQC-NMR**

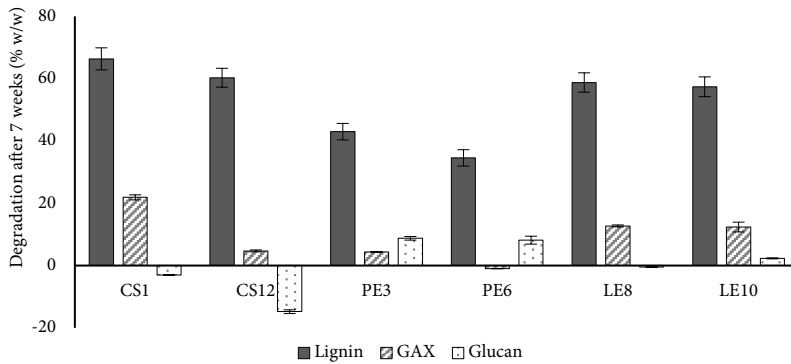
Gijs van Erven, Nazri Nayan, Anton S.M. Sonnenberg, Wouter H. Hendriks, John W. Cone and Mirjam A. Kabel

**Table 6-S1.** Identity, structural classification and relative abundance of lignin-derived pyrolysis products by <sup>13</sup>C-IS Py-GC/MS. Control and fungal-treated wheat straw samples after 7 weeks of treatment. CS *Ceriporiopsis subvermispora*, PE *Pleurotus eryngii*, LE *Lentinula edodes*. Average of triplicates.

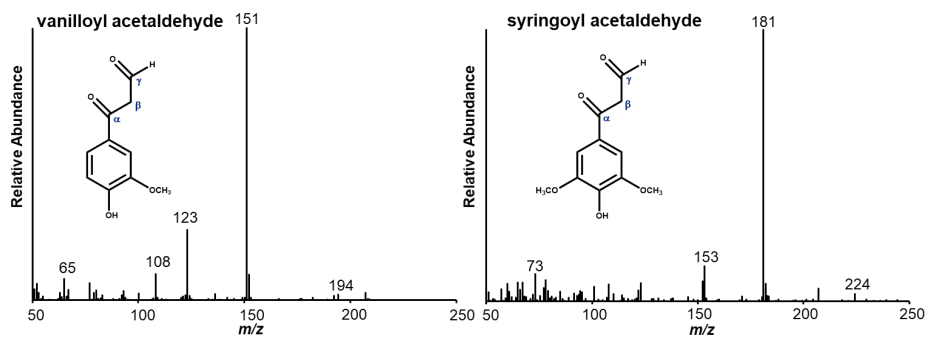
#	Compound	Ret. time (min)	Structural feature	Sidechain length	M <sub>w</sub> <sup>12</sup> C (g·mol <sup>-1</sup> )	Control	CS1	CS12	PE3	PE6	LE8	LE10
1	phenol	10.03	H, unsub.	0	94	0.7	2.0	1.9	1.1	0.9	1.5	1.6
2	guaiacol	10.31	G, unsub.	0	124	2.1	4.2	4.0	2.7	2.5	3.2	3.4
3	2-methylphenol	11.29	H, methyl	C <sub>α</sub>	108	0.2	0.4	0.4	0.2	0.2	0.3	0.3
4	4-methylphenol (+3-MP)	12.23	H, methyl	C <sub>α</sub>	108	0.5	1.1	1.2	0.8	0.7	0.9	1.0
5	4-methylguaiacol	13.01	G, methyl	C <sub>α</sub>	138	0.7	1.0	1.0	0.9	0.9	0.9	0.9
6	2,4-dimethylphenol	13.46	H, methyl	C <sub>α</sub>	122	0.1	0.2	0.2	0.1	0.1	0.1	0.2
7	4-ethylphenol	14.52	H, ethyl	C <sub>β</sub>	122	0.1	0.1	0.1	0.1	0.1	0.1	0.1
8	4-ethylguaiacol	15.19	G, ethyl	C <sub>β</sub>	152	0.1	0.2	0.2	0.2	0.2	0.2	0.2
9	4-vinylguaiacol	16.64	G, vinyl	C <sub>β</sub>	150	20.3	20.4	20.3	19.9	21.4	21.7	21.0
10	4-vinylphenol	16.77	H, vinyl	C <sub>β</sub>	120	7.8	7.7	7.8	7.3	7.8	7.8	7.9
11	eugenol	17.26	G, misc.	C <sub>γ</sub>	164	0.2	0.2	0.2	0.2	0.2	0.2	0.2
12	4-propylguaiacol	17.34	G, misc.	C <sub>γ</sub>	166	0.1	0.2	0.2	0.2	0.2	0.2	0.2
13	syringol	18.00	S, unsub.	0	154	1.9	3.2	3.5	2.3	2.3	2.4	2.7
14	cis-iso Eugenol	18.63	G, misc.	C <sub>γ</sub>	164	0.1	0.1	0.1	0.1	0.1	0.1	0.1
15	4-propenylphenol	19.54	H, misc.	C <sub>γ</sub>	134	0.1	0.1	0.1	0.1	0.1	0.1	0.1
16	trans-iso Eugenol	19.90	G, misc.	C <sub>γ</sub>	164	0.8	1.0	0.9	1.1	1.1	1.1	1.1
17	4-methylsyringol	20.26	S, methyl	C <sub>α</sub>	168	0.5	0.6	0.6	0.6	0.6	0.6	0.6
18	vanillin	20.35	G, C <sub>α</sub> -O	C <sub>α</sub>	152	1.2	2.1	2.3	1.4	1.2	1.4	1.4
19	4-propylneguaiacol	20.64	G, misc.	C <sub>γ</sub>	162	0.1	0.1	0.1	0.1	0.1	0.1	0.1
20	4-alleneguaiacol	20.90	G, misc.	C <sub>γ</sub>	162	0.1	0.1	0.1	0.1	0.1	0.1	0.1
21	homovanillin	21.81	G, C <sub>β</sub> -O	C <sub>β</sub>	166	0.5	0.7	0.8	0.5	0.5	0.5	0.5
22	4-ethylsyringol	22.00	S, ethyl	C <sub>β</sub>	182	0.0	0.0	0.1	0.1	0.0	0.0	0.0
23	acetovanillone	22.27	G, C <sub>α</sub> -O	C <sub>β</sub>	166	0.3	1.1	1.2	0.6	0.5	0.6	0.7
24	4-hydroxybenzaldehyde	23.01	H, C <sub>α</sub> -O	C <sub>α</sub>	122	0.2	0.3	0.3	0.2	0.1	0.2	0.2
25	4-vinylsyringol	23.32	S, vinyl	C <sub>β</sub>	180	2.4	2.0	2.2	2.3	2.4	2.3	2.3
26	guaiacylacetone	23.50	G, C <sub>β</sub> -O	C <sub>γ</sub>	180	0.3	0.7	0.7	0.4	0.3	0.4	0.4
27	4-allylsyringol	23.75	S, misc.	C <sub>γ</sub>	194	0.2	0.2	0.2	0.2	0.2	0.2	0.2
28	propiovanillone	24.18	S, C <sub>α</sub> -O	C <sub>γ</sub>	180	0.0	0.1	0.1	0.0	0.0	0.1	0.1
29	guaiacyl vinyl ketone	24.47	G, C <sub>α</sub> -O	C <sub>β</sub>	178	0.1	0.2	0.2	0.1	0.1	0.1	0.1
30	vanilloyl acetaldehyde	24.69	G, C <sub>α</sub> -O, C <sub>γ</sub> -O	C <sub>γ</sub>	194	0.2	4.3	3.9	0.9	0.6	1.5	1.5
31	cis-4-propenylsyringol	24.88	S, misc.	C <sub>γ</sub>	194	0.1	0.1	0.1	0.1	0.1	0.1	0.1
32	4-propynesyringol	25.52	S, misc.	C <sub>γ</sub>	192	0.1	0.1	0.1	0.1	0.2	0.1	0.1
33	4-allenesyringol	25.73	S, misc.	C <sub>γ</sub>	192	0.1	0.1	0.1	0.1	0.1	0.1	0.1
34	trans-4-propenylsyringol	26.19	S, misc.	C <sub>γ</sub>	194	0.9	0.8	0.9	1.0	0.6	0.9	0.9
35	dihydroconiferyl alcohol	26.22	S, C <sub>γ</sub> -O	C <sub>γ</sub>	182	0.1	0.1	0.1	0.1	0.1	0.1	0.1
36	syringaldehyde	26.75	S, C <sub>α</sub> -O	C <sub>α</sub>	182	0.7	0.8	1.0	0.7	0.7	0.6	0.7
37	cis-coniferyl-alcohol	26.82	G, C <sub>γ</sub> -O	C <sub>γ</sub>	180	1.3	1.1	1.0	1.4	1.3	1.3	1.3
38	homosyringaldehyde	27.75	S, C <sub>γ</sub> -O	C <sub>γ</sub>	196	0.3	0.4	0.5	0.3	0.3	0.3	0.3
39	acetosyringone	28.20	S, C <sub>α</sub> -O	C <sub>β</sub>	196	0.6	1.2	1.4	0.9	0.8	0.8	0.8
40	trans-coniferyl alcohol	28.55	G, C <sub>γ</sub> -O	C <sub>γ</sub>	180	31.6	22.4	20.0	30.0	29.8	28.3	27.7
41	trans-coniferaldehyde	28.92	G, C <sub>γ</sub> -O	C <sub>γ</sub>	178	1.8	2.0	1.8	2.1	1.9	2.0	1.9
42	syringylacetone	29.13	S, C <sub>β</sub> -O	C <sub>γ</sub>	210	0.3	0.7	0.8	0.4	0.4	0.5	0.5
43	propiosyringone	29.75	S, C <sub>α</sub> -O	C <sub>γ</sub>	210	0.0	0.1	0.1	0.1	0.1	0.0	0.1
44	syringoyl acetaldehyde	29.90	S, C <sub>α</sub> -O, C <sub>γ</sub> -O	C <sub>γ</sub>	224	0.2	3.4	4.0	1.0	0.9	1.1	1.4
45	syringyl vinyl ketone	30.03	S, C <sub>α</sub> -O	C <sub>γ</sub>	208	0.0	0.1	0.1	0.1	0.1	0.0	0.0
46	dihydrosinapyl alcohol	30.22	G, C <sub>γ</sub> -O	C <sub>γ</sub>	212	0.0	0.0	0.0	0.0	0.0	0.0	0.0
47	cis-sinapyl alcohol	32.07	S, C <sub>γ</sub> -O	C <sub>γ</sub>	210	0.9	0.6	0.6	0.8	0.8	0.7	0.7
48	trans-sinapyl alcohol	33.79	S, C <sub>γ</sub> -O	C <sub>γ</sub>	210	16.6	9.3	10.0	13.6	13.7	11.6	11.9
49	trans-sinapaldehyde	34.00	S, C <sub>γ</sub> -O	C <sub>γ</sub>	208	2.0	2.3	2.4	2.4	2.3	2.1	2.0



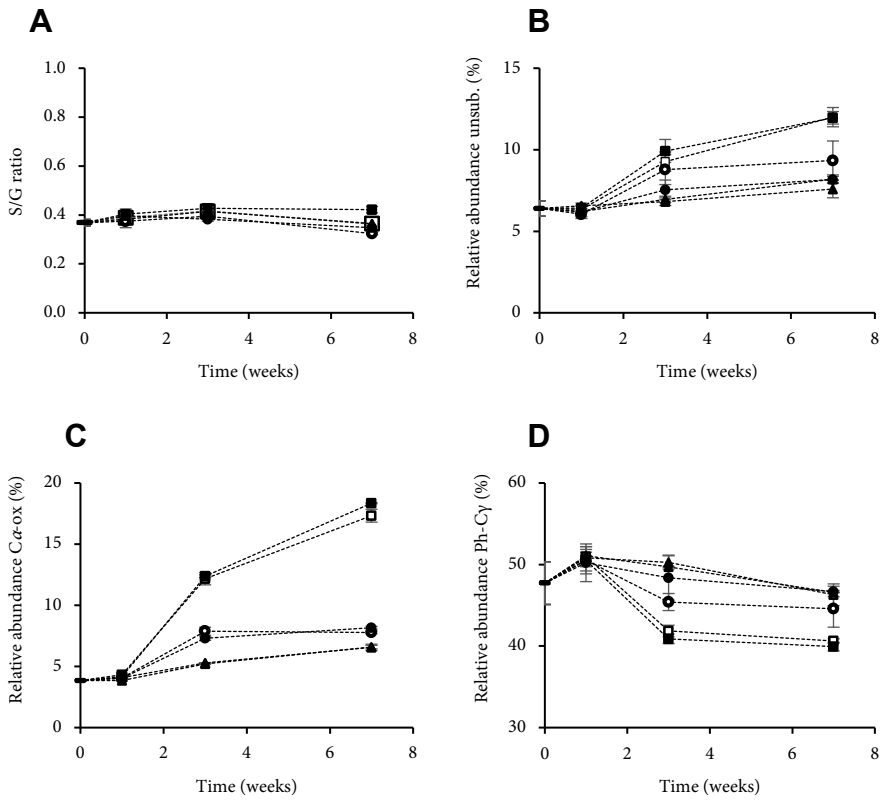
**Fig. 6-S1.** Residual lignin, glucuronoarabinoxylan (GAX) and glucan (cellulose) in fungal-spent wheat straw during growth (1, 3 and 7 weeks) of *C. subvermispota* (CS), *P. eryngii* (PE) and *L. edodes* (LE) strains. Average and standard deviation of triplicates.



**Fig. 6-S2.** Lignin, glucuronoarabinoxylan (GAX) and glucan (cellulose) degradation after 7 weeks of fungal growth. Average and standard deviation of triplicates.



**Fig. 6-S3.** EI-MS (70 eV) spectra and structures of vanilloyl acetaldehyde (VAL) and syringoyl acetaldehyde (SAL) observed by Py-GC/MS. Compounds were identified on the basis of fragmentation pattern and retention time. Average mass spectrum across the chromatographic peak with noise subtraction at two sides.



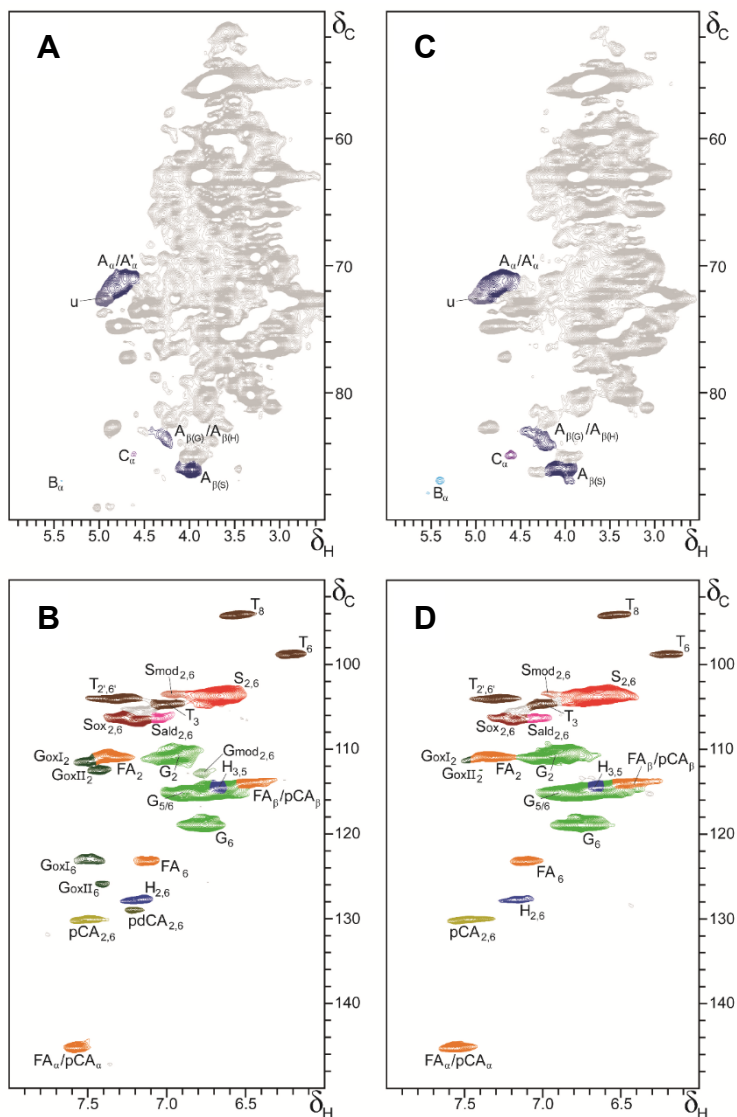
**Fig. 6-S4.** Structural features of fungal-spent wheat straw lignin during growth (1, 3 and 7 weeks) of *C. subvermispota* (CS), *P. eryngii* (PE) and *L. edodes* (LE) strains as determined by semi-quantitative Py-GC/MS. S/G ratio (A) and relative abundances of unsubstituted (B), C $\alpha$ -oxidized (C) and Ph-C $\gamma$  (D) substructures are based on the structural classification shown in Supplementary Information Table 6-S1.  $\square$  CS1,  $\blacksquare$  CS12,  $\circ$  PE3,  $\bullet$  PE6,  $\triangle$  LE8,  $\blacktriangle$  LE10. Average and standard deviation of triplicates.

**Table 6-S3.** Assignments of the lignin  $^{13}\text{C}$ - $^1\text{H}$  correlation peaks in the HSQC spectra of untreated and fungal-spent wheat straw. (t) Tentative assignment. Assignment by comparison with literature\*.

Label	$\delta/\delta_{\text{H}}$ (ppm)	Assignment
A $_{\alpha}$ (G)	70.9/4.70	C $_{\alpha}$ -H $_{\alpha}$ in $\beta$ -O-4' substructures linked to G-unit
A $_{\alpha}$ (S)	71.8/4.82	C $_{\alpha}$ -H $_{\alpha}$ in $\beta$ -O-4' substructures linked to S-unit
A $_{\beta}$ (G)	83.5/4.25	C $_{\beta}$ -H $_{\beta}$ in $\beta$ -O-4' substructures linked to G-unit
A $_{\beta}$ (S)	85.9/4.09	C $_{\beta}$ -H $_{\beta}$ in $\beta$ -O-4' substructures linked to S-unit
C $_{\alpha}$	84.9/4.64	C $_{\alpha}$ -H $_{\alpha}$ in resinol substructures
B $_{\alpha}$	86.9/5.42	C $_{\alpha}$ -H $_{\alpha}$ in phenylcoumaran substructures
T $_{8}$	94.1/6.56	C $_{8}$ -H $_{8}$ in triclin
T $_{6}$	98.8/6.20	C $_{6}$ -H $_{6}$ in triclin
Smod $_{2,6}$	103.5/6.97	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in S-unit derivative (t)
S $_{2,6}$	103.6/6.68	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in S-unit
T $_{2'}$	104.0/7.31	C $_{2'}$ -H $_{2'}$ and C $_{6'}$ -H $_{6'}$ in triclin
T $_{3}$	104.6/6.99	C $_{3}$ -H $_{3}$ in triclin
Sox $_{2,6}$	106.4/7.32	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in C $_{\alpha}$ -oxidized (C $_{\alpha}$ =O) S-unit
Sox $_{2,6}$	106.5/7.18	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in C $_{\alpha}$ -oxidized (C $_{\alpha}$ OOH) S-unit
Sald $_{2,6}$	106.3/7.03	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in cinnamaldehyde end-group S-unit
G $_{2}$	110.5/6.94	C $_{2}$ -H $_{2}$ in G-unit
FA $_{2}$	110.9/7.32	C $_{2}$ -H $_{2}$ in ferulate
GoxI $_{2}$	111.4/7.53	C $_{2}$ -H $_{2}$ in C $_{\alpha}$ -oxidized G-unit
GoxII $_{2}$	112.4/7.42	C $_{2}$ -H $_{2}$ in C $_{\alpha}$ -oxidized G-unit (t)
Gmod $_{2}$	112.7/6.76	C $_{2}$ -H $_{2}$ in G-unit derivative (t)
FA $_{\beta}$ /pCA $_{\beta}$	113.9/6.51	C $_{\beta}$ -H $_{\beta}$ in ferulate/p-coumarate (and derivatives)
H $_{3,5}$ /FA $_{5}$	114.5/6.69	C $_{3}$ -H $_{3}$ and C $_{5}$ -H $_{5}$ in H-unit, C $_{5}$ -H $_{5}$ in FA
G $_{5}$ /G $_{6}$ /pCA $_{3,5}$	115.1/6.94 and 115.3/6.72	C $_{5}$ -H $_{5}$ and C $_{6}$ -H $_{6}$ in G-unit, C $_{3}$ -H $_{3}$ and C $_{5}$ -H $_{5}$ of pCA
G $_{5}$	118.9/6.77	C $_{5}$ -H $_{5}$ in G-unit
GoxI $_{6}$	122.8/7.49	C $_{6}$ -H $_{6}$ in C $_{\alpha}$ -oxidized G-unit
FA $_{6}$	123.1/7.12	C $_{6}$ -H $_{6}$ in ferulate
GoxII $_{6}$	125.8/7.40	C $_{2}$ -H $_{2}$ in C $_{\alpha}$ -oxidized G-unit (t)
H $_{2,6}$	127.7/7.17	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in H-unit
pdCA $_{2,6}$	129.0/7.20	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in <i>p</i> -dihydrocoumarate
pCA $_{2,6}$	130.1/7.48	C $_{2}$ -H $_{2}$ and C $_{6}$ -H $_{6}$ in <i>p</i> -coumarate
FA $_{\alpha}$ /pCA $_{\alpha}$	145.0/7.56	C $_{\alpha}$ -H $_{\alpha}$ in ferulate/p-coumarate

\* Das *et al.* (2018); Del Río *et al.* (2012, 2015); Ralph *et al.* (2009b); Rencoret *et al.* (2010, 2017); Villaverde *et al.* (2009); Yuan *et al.* (2011). Refer to the main text for full references.





**Fig. 6-S5.** HSQC NMR spectra of fungal-spent wheat straw. CS12 (A,B), PE3 (C,D), PE6 (E,F) LE8 (G,H) LE10 (I,J). A,C,E,G,I: aliphatic ( $\delta_C/\delta_H$  50-90/2.5-6.0) region, B,D,F,H,J: aromatic/unsaturated ( $\delta_C/\delta_H$  90-160/6.0-8.0) region. Structures of annotated correlation peaks are presented in Fig. 5.  $G_{mod}$  and  $S_{mod}$  are presented in a lighter colour of G and S, respectively. Carbohydrate and unassigned signals are presented in gray. u: unassigned signal overlapping  $A_\alpha/A'_\alpha$ .

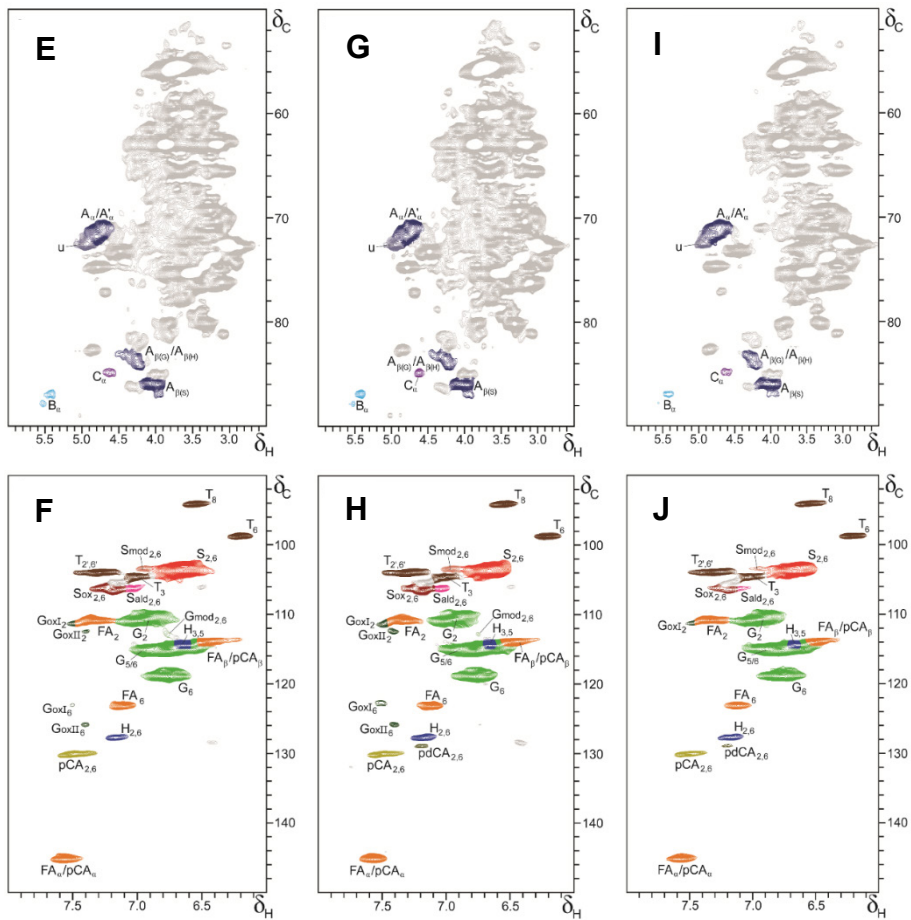


Fig. 6-S5. (continued)



“

If you can, help others; if you cannot do that, at least do not harm them.

”

- DALAI LAMA

# Improving ruminal digestibility of various wheat straw types by white-rot fungi

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## ABSTRACT

The present study investigated the ruminal degradability of various wheat straw types by the white-rot fungi *Ceriporiopsis subvermispota* (CS) and *Lentinula edodes* (LE). Hereto, wheat straw of different maturity stages (MS) and cultivars (CV), were treated with the fungi for 7 weeks and assessed for chemical composition and *in vitro* gas production (IVGP). Both fungi showed more pronounced degradation of lignin on a more mature straw (MS3; 89.0%), as compared to the straw harvested at an earlier stage (MS1; 70.7%). Quantitative <sup>13</sup>C-IS Py-GC/MS revealed that lignin in more mature straw was degraded and modified to larger extents. In contrast, cellulose was less degraded in MS3, as compared to MS1 (~8.3 vs. 14.6%). There was no effect of different MS on the IVGP of the fungal-treated straws. Of different straw cultivars, the extent of lignin degradation varied greatly (47 to 93.5%). This may explain the significant ( $P < 0.001$ ) effect of cultivar on the IVGP of the fungal-treated straws. Regardless of the factors tested, both fungi were highly capable to improve the IVGP of all straw types by 15.3 to 47.6%, (as compared to untreated straw), with CS performing better than LE – either on different MS (~33.6% vs. 20.4%) or CVs (~43.2 vs. 29.1%). The extent of lignin degradation caused by fungal treatment was more pronounced on the more mature and lignified straw, while variable results were obtained with different cultivars. Both fungi were capable of improving the IVGP of various straw types.

**Keywords:** White-rot fungi; wheat straw cultivar; wheat straw maturity; *in vitro* gas production; ruminant feed; lignocellulosic biomass.

## INTRODUCTION

Valorization of lignocellulosic biomass, such as wheat straw, as an alternative feed ingredient is important for a sustainable animal production. To unlock the energy potential of lignocellulose, various physicochemical methods have been used, such as hydrothermal treatment, ammonia fiber expansion and the use of acids and alkaline media (Bals *et al.*, 2010; Hendriks and Zeeman, 2009; Sarnklong *et al.*, 2010). These methods are used to increase the enzymatic accessibility of the structural carbohydrates by breaking the lignin barrier and thus improving the degradability of the feedstock by ruminants. Although they are attractive for industrial-scale applications, due to time efficiency, the potential adverse impact on the environment is a major concern (Isroi *et al.*, 2011). Therefore, biological pretreatments, particular using fungi, have become a favorable approach (Nayan *et al.*, 2017; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015a). Some strains of white-rot fungi possess unique capabilities in degrading highly-recalcitrant lignocellulosic biomass, whereby lignin is being selectively degraded, increasing the available carbohydrate contents of the biomass.

The effectiveness of a fungal pretreatment can be influenced by many factors, such as fungal strain, substrate and culture conditions (Tuyen *et al.*, 2013; Van Kuijk *et al.*, 2015a, 2015b). Variability in substrate and culture conditions used, leads to difficulties in comparing the effectiveness of fungi across different studies (Van Kuijk *et al.*, 2015b). The quality of the straw, i.e. ratio of lignin to total carbohydrates, is not only an important factor influencing its digestibility in the rumen (Schiere *et al.*, 2004), but may also affect the effectiveness of a fungal pretreatment (Van Kuijk *et al.*, 2015b). Unfortunately, standardizing the straw for an optimal result is difficult and impractical, considering disparities among the straw types and conditions, as well as the differences in post-harvest residue management (Snyder *et al.*, 2009). The present study was conducted to assess the effect of straw quality on lignocellulose degradation by fungi and the subsequent effect on ruminal degradability of the treated straw.

Straw quality is affected by many factors – among others, maturity stage, cultivar and other factors related to management and environment (Labuschagne *et al.*, 2000; Moore and Jung, 2001). The maturity of the plant, in particular, correlates with the extent of lignin deposition in the plant tissues (Moore and Jung, 2001). The total fiber content increases as the plant matures (lower leaf to stem ratio (Martiniello *et al.*, 1997)) and also the lignin, cellulose and hemicellulose composition changes. As straw consists mainly of fiber (~85%), changes in the cell wall composition with maturity may thus have an influence on the lignocellulose degradation by fungi. Another important factor is the

wheat straw genotype. In wheat breeding, nutrient-use efficiency and disease resistance are among the selection parameters, in contrast to straw quality (Labuschagne *et al.*, 2000; Lammerts Van Bueren *et al.*, 2011).

Recently, high potential strains of the white-rot fungi species *Ceriporiopsis subvermispora* and *Lentinula edodes* have been selected for the bioprocessing of wheat straw into ruminant feed (Nayan *et al.*, 2018). To determine if these high potential strains could perform well across different straw types, two independent trials were carried out in the present study. These trials investigated the effect of two main sources of straw variation: (1) different maturity of the straw around harvest and (2) different straw cultivars, on the fungal growth and their persistency in improving the *in vitro* degradability of the straw. Also the lignin degradation characteristics of wheat straw with different maturities were assessed, using pyrolysis coupled to gas chromatography with mass spectrometry (Py-GC/MS) (Van Erven *et al.*, 2017).

## MATERIALS AND METHODS

### Effect of different maturity stages

#### *Harvesting wheat straw with different maturities*

Wheat straw (*Triticum aestivum* L.) of the same variety (*Quintus*) was harvested during the summer of 2015 from the experimental field UNIFARM of Wageningen University. Each harvest covered a plot size of 8.5 × 1.5 m. Zadoks *et al.* (1974) codes were used to characterize the following maturity stages of the wheat plant. The first harvest was carried out on 14 July (MS1, code 83 – soft dough development, 37.3% dry weight), second harvest on 28 July (MS2, code 87 – hard dough development, 41.2% dry weight) and the third harvest on 11 August (MS3, code 91 – ripening, 64.6% dry weight). The wheat plants were cut at 10 cm above the ground and the fresh plants were dried on a drying panel for a week (~60°C). Prior to chopping, the spike containing the wheat grains was removed, leaving the straw (leaves and stalk) intact. The trimmed straw was chopped into approximately 3 cm pieces.

#### *Fungal pretreatment of the wheat straw*

The cultures of the white-rot fungi *C. subvermispora* (CS; CBS 347.63) and *L. edodes* (LE; sh 03/08) were maintained on malt agar extract, containing (g L<sup>-1</sup>): 20.0 of malt extract, 0.5 of KH<sub>2</sub>PO<sub>4</sub>, 0.5 of MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.5 of Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O with pH 5.4 at 24°C. Once the fungi fully colonized the agar surface, spawn was prepared by placing a



piece of agar culture (1.5×2.0 cm) into sterilized (121°C, 20 min) sorghum grains. The spawn was incubated at 24°C for 4 or 5 weeks. The chopped straw was soaked in water for 3 d at room temperature and excess water was drained off for 5 h. Adjustments were made based on the final moisture content of the straw – which ranged from 77 (MS1) to 80% (MS3), for each container (172×110×70 mm; Combiness, Nevele, Belgium) to contain  $41.7 \pm 0.3$  g of dry matter. After autoclaving at 121°C for 1 h, the straw was aseptically inoculated with the previously prepared spawn at 10% of the dry weight. Control (untreated) and fungal-treated wheat straw were incubated in triplicate under solid state fermentation at 24°C for 7 weeks in a climate-controlled chamber. All weekly samples were freeze-dried and ground over a 1 mm sieve using a cross beater mill (100AN, Peppink, Olst, the Netherlands).

### *Chemical analysis*

Each sample was analyzed for dry matter (DM; ISO 6496, 1999) and ash (ISO 5984, 2002) content. Crude protein was calculated by multiplying the nitrogen content (ISO 5983, 2005) by 6.25. The Van Soest *et al.* (1991) method was used to determine the cell wall composition. Neutral detergent fiber (NDF) was determined using a heat-stable amylase (thermamy) and alcalase; acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined by boiling the sample in an acid detergent solution, and the latter was further treated with 72% (v/v) H<sub>2</sub>SO<sub>4</sub>. All fiber contents were corrected for ash. Hemicellulose was calculated as the difference between NDF and ADF, while cellulose was calculated as the difference between ADF and ADL. Reducing sugars in the ethanol-extract of the straw were determined by measuring the oxidation reaction between the hydrolyzed monosaccharides with copper (II) and neocuproine at 460 nm. Absolute amounts (g) of each component were calculated from the remaining amount (g) of the freeze-dried samples.

### *Ergosterol estimation*

Fungal biomass was estimated by determining the content of ergosterol. Details of the procedure have previously been described (Nayan *et al.*, 2017; Niemenmaa *et al.*, 2008). Samples (~200 mg) were saponified with 10% (1:9) KOH/methanol solution at 80°C for 60 min. After cooling, the ergosterol was extracted from the samples through a series of mixing with 1 ml water and 2 ml hexane. The hexane layers from the same sample were collected by centrifuging and pooled into a tube. The pooled hexane layer was dried in a vacuum evaporation system (Rapidvap, Kansas, MO, USA) before re-dissolving in

methanol. The solution was filtered into a high performance liquid chromatography (HPLC) vial for Waters HPLC-PDA analysis (Alliance HPLC system, Milford, USA). Cholecalciferol (vitamin D<sub>3</sub>) was used as an internal standard. The ergosterol peak was detected at 280 nm.

#### *In vitro gas production (IVGP)*

The *in vitro* gas production (IVGP) was used to assess the ruminal degradability of the wheat straw. The gas production experiment was performed according to the procedures described by Cone *et al.* (1996). In brief, rumen fluid was collected from 2 non-lactating cows fed 1 kg concentrate and grass silage ad libitum. The rumen fluid was filtered through a cheesecloth and mixed (1:2 v/v) with phosphate-bicarbonate buffer solution, which also contained trace elements, hydrolyzed casein, redox indicator and reducing agent (Cone *et al.*, 1996). The samples (0.5 g) were incubated in 60 ml of buffered rumen fluid for 72 h and the gas production was automatically registered. The gas production data were fitted to a biphasic model (Groot *et al.*, 1996) to determine the kinetic parameters ( $A_n$ ,  $B_n$ ,  $C_n$ ,  $t_{R_{mn}}$ ,  $R_{mn}$ ), where  $n$  is the phase number (1 or 2).  $A_n$  is the asymptotic gas production (ml g<sup>-1</sup> OM) of phase  $n$ ;  $B_n$  is the half time of the maximum gas production (h);  $C_n$  is a parameter to determine the steepness of the curve;  $t_{R_{mn}}$  is the time of the maximum fractional rate of substrate degradation (h); and  $R_{mn}$  the maximum fractional rate of substrate degradation (h<sup>-1</sup>).

#### *Quantitative pyrolysis GC/MS with <sup>13</sup>C lignin as internal standard*

Prior to Py-GC/MS, ground wheat straw (1 mm) was ball-milled in a MM200 mixer mill (Retsch, Haan, Germany) and biological triplicates were mixed to one replicate. Pyrolysis of the ball-milled sample was carried out as previously described in detail (Van Erven *et al.*, 2017). Briefly, 10 µL of a <sup>13</sup>C lignin internal standard (IS) solution (1 mg ml<sup>-1</sup>) was mixed with ~80 µg of sample and dried before analysis. Lignin-derived pyrolysis products were monitored in full MS mode on the two most abundant fragments per compound (both <sup>12</sup>C and <sup>13</sup>C). Area for each compound was normalized by dividing by the respective relative response factor (RRF). RRF values were updated to system performance by recalculation to obtain an identical relative abundance of lignin-derived pyrolysis products of the <sup>13</sup>C IS added to a wheat straw reference sample. Lignin content (% w/w) was determined as the sum of lignin-derived pyrolysis products, where RRF corrected areas for each compound were multiplied with the molecular weight of the respective compound and summed instead of the application of the published correction

factor of 1.057 (Van Erven *et al.*, 2017). Relative abundances of lignin-derived pyrolysis products were based on areas normalized for the  $^{13}\text{C}$  analogues from the IS present in the same sample to distinguish matrix and treatment effects. Areas were not corrected for molecular weight before relative abundance determination as previously described by Del Río *et al.* (2012), as RRF values are mole-based. Compounds were classified according to their structural feature (Table 7-S1) and summed. All samples were prepared and analyzed in triplicate.

### Effect of different wheat cultivars

An independent experiment of assessing the effects of different straw cultivars on the fungal pretreatment was carried out. Wheat straw from five winter wheat cultivars – *Britannia* (CV1), *Cellule* (CV2), *Henrik* (CV3), *Residence* (CV4) and *Tabasco* (CV5), were purchased from Limagrain (Rilland, the Netherlands). Any remaining spike from the straw was removed prior to chopping. The wheat straw was chopped at 3 cm length. The chopped straw underwent the same processing and treatment procedures as previously described with the same fungal strains used as mentioned above. Solid state fermentation of the inoculated wheat straw was carried out at 24°C for 7 weeks in a climate controlled room with minimum exposure to light. The weekly samples were weighed, freeze-dried and ground over a 1 mm sieve for further analysis. All samples were subjected to the same chemical and ergosterol analyses as well as IVGP procedure as described above. No Py-GC/MS was conducted on these samples.

### Statistics

Data from both experiments were independently analyzed by analysis of variance using the generalized linear model (GLM) in SAS 9.3, followed by post-hoc multiple comparison using least significance differences. The statistical model for the ergosterol and IVGP data included the effect of levels in each factor (maturity stage 1 to 3; or cultivar 1 to 5), treatment (control, *C. subvermispota* and *L. edodes*), week and the interaction effects of all three terms. For chemical analyses, the end point data were analyzed by excluding week and its interaction terms from the previous model. Analysis of pyrolysis data of different maturity stages reflected analytical rather than biological variances as three biological replicates of the same treatment (with an equal amount) were mixed prior to the analysis. Minimum significance threshold level was set at  $P < 0.05$ . Pearson's Product-Moment Correlation ( $r$ ) coefficients were also determined among the measured variables.

## RESULTS AND DISCUSSION

### Effect of different maturity stages

#### *Mass balances for the wheat straw at different maturities*

The dry matter, ash content and the mass balances of different wheat straw maturities treated with CS and LE for 7 weeks are reported in Table 7.1. The water holding capacity of the straw was increased with straw maturity, as evident by a 7% lower DM content of MS3 straw as compared to MS1 and MS2 (Table 7.1). However, the total amounts of OM were not different for the untreated straws at different maturities. To allow a comparison across different maturity stages, the amounts of the quantified nutrients were expressed per 100 g of starting OM for the respective stages. For the untreated straws, higher amounts of ADL (12.2%) and cellulose (6%) were seen with increasing straw maturity. The untreated MS3 straw contained a significantly ( $P < 0.01$ ) higher amount of ADL as compared to the rest. Hemicellulose was significantly ( $P < 0.001$ ) lower in the MS3 straw as compared to its counterparts, while high ( $P < 0.01$ ) amounts of free sugars and crude protein were observed for the MS1 straw.

There were significant ( $P < 0.001$ ) losses of OM after 7 weeks of fungal pretreatment (17.5 to 25.3%), with high losses observed for fungal-treated MS1 straw. LE resulted in a significantly ( $P < 0.01$ ) higher loss of OM compared to CS at any maturity stage. Overall, both fungi significantly ( $P < 0.001$ ) degraded all cell wall components and increased the amount of soluble sugars. A lesser amount of cellulose was degraded when both fungi grew on MS3 compared to MS1 (~8.3 vs. 14.6%, respectively). Interestingly, lignin degradation by both fungi was more pronounced on mature straw. The delignification by CS, for instance, was similar when grown on MS1 and MS2 straw (~82%), whereas on MS3 straw, CS degraded 98.2% of the total amount of ADL. The figures for lignin degradation presented here, were higher than that in previous reports (33 to 60%), which were carried out under similar treatment conditions (Nayan *et al.*, 2017; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015a), but at an unknown stage of maturity. Meanwhile, a similar trend was also observed for the hemicellulose with a higher percentage of degradation (66.2 to 79.3%) for both fungi. CS consistently resulted in a higher degradation of ADL and hemicellulose compared to LE for all straw maturities. In addition, both fungi also increased ( $P < 0.001$ ) the amount of free sugars in the treated straw, which may arise from the breakdown of the cell wall polysaccharides. The apparent susceptibility of a more mature straw to fungal delignification is of particular interest here. To investigate the delignification characteristics of both fungi, the samples from the control (week 0) and

**Table 7.1.** Dry matter, ash content and mass balances for wheat straw of different maturity stages, treated with *C. subvermispora* (CS) and *L. edodes* (LE) for 7 weeks.

Maturity stage <sup>†</sup>	Treatment	DM (g kg <sup>-1</sup> )	Ash (g kg <sup>-1</sup> DM)	Amount (g per 100 g of starting OM) <sup>‡</sup>						
				OM	Cell	Hcell	ADL	Sugar	CP	NI
MS1	Control	184.3 <sup>a</sup>	18.9 <sup>d</sup>	100.0 <sup>a</sup>	47.7 <sup>b</sup>	34.4 <sup>a</sup>	7.4 <sup>c</sup>	0.7 <sup>d</sup>	3.3 <sup>b</sup>	6.6 <sup>d</sup>
	CS	158.4 <sup>c</sup>	24.7 <sup>ab</sup>	78.7 <sup>c</sup>	40.9 <sup>de</sup>	8.3 <sup>c</sup>	1.4 <sup>f</sup>	1.8 <sup>bc</sup>	3.5 <sup>a</sup>	22.7 <sup>a</sup>
	LE	156.9 <sup>c</sup>	23.6 <sup>b</sup>	74.7 <sup>d</sup>	40.5 <sup>e</sup>	11.6 <sup>c</sup>	2.9 <sup>d</sup>	1.8 <sup>bc</sup>	3.2 <sup>bc</sup>	14.5 <sup>c</sup>
MS2	Control	183.3 <sup>a</sup>	20.7 <sup>c</sup>	100.0 <sup>a</sup>	49.6 <sup>a</sup>	34.1 <sup>a</sup>	7.8 <sup>b</sup>	0.5 <sup>e</sup>	2.5 <sup>e</sup>	5.5 <sup>d</sup>
	CS	154.5 <sup>cd</sup>	25.5 <sup>ab</sup>	82.5 <sup>b</sup>	42.3 <sup>d</sup>	8.7 <sup>e</sup>	1.4 <sup>f</sup>	2.1 <sup>a</sup>	3.1 <sup>c</sup>	24.8 <sup>a</sup>
	LE	150.7 <sup>d</sup>	24.3 <sup>ab</sup>	79.0 <sup>c</sup>	44.4 <sup>c</sup>	10.8 <sup>cd</sup>	2.2 <sup>e</sup>	1.9 <sup>b</sup>	2.8 <sup>d</sup>	16.9 <sup>b</sup>
MS3	Control	171.3 <sup>b</sup>	19.9 <sup>cd</sup>	100.0 <sup>a</sup>	50.7 <sup>a</sup>	30.8 <sup>b</sup>	8.4 <sup>a</sup>	0.5 <sup>e</sup>	2.6 <sup>e</sup>	7.0 <sup>d</sup>
	CS	150.9 <sup>d</sup>	25.8 <sup>a</sup>	82.2 <sup>b</sup>	46.2 <sup>b</sup>	6.4 <sup>f</sup>	0.2 <sup>g</sup>	2.1 <sup>a</sup>	2.9 <sup>d</sup>	24.5 <sup>a</sup>
	LE	145.3 <sup>e</sup>	25.3 <sup>a</sup>	78.5 <sup>c</sup>	46.8 <sup>b</sup>	9.9 <sup>d</sup>	1.7 <sup>f</sup>	1.7 <sup>c</sup>	2.8 <sup>d</sup>	15.7 <sup>bc</sup>
RMSE		2.36	0.96	0.74	0.89	0.67	0.23	0.06	0.09	1.27

Values with different superscripts within a column are significantly ( $P < 0.05$ ) different. DM: dry matter; OM: organic matter; Cell: cellulose; Hcell: hemicellulose; ADL: acid detergent lignin; Sugar: free reducing sugar from ethanol-extract of wheat straw; CP: crude protein ( $N \times 6.25$ ); NI: unaccounted OM; RMSE: Root mean square error.

<sup>†</sup> Maturity stages around harvest with 2 weeks differences among MS1, MS2 and MS3.

<sup>‡</sup> Calculated from the remaining materials for each treatment (g), using respective starting OM for each maturity stage.

after 7 weeks of fungal pretreatment were subjected to quantitative  $^{13}\text{C}$ -IS Py-GC/MS analysis. There was an average variation of 8% in the ADL measurement of biological replicates of the same treatment. Hence, prior to Py-GC/MS, the biological replicates were thoroughly mixed to allow analytical triplicates for detailed and more accurate lignin analysis.

#### *Quantitative $^{13}\text{C}$ -IS Py-GC/MS of the wheat straw*

A total of 34 lignin-derived compounds were released upon pyrolysis and monitored (see supporting information Table 7-S1). The lignin content as quantified using  $^{13}\text{C}$ -IS Py-GC/MS and its structural features are summarized in Table 7.2. Overall, the untreated straws showed comparable amounts of lignin (~23.8 g per 100 g OM), which were in contrast to the previous ADL method. It is inferred that the mature straw may contain high amount of recalcitrant residual lignin that was retained in the ADL fraction, which explains a higher ADL in MS3 straw, compared to its counterparts. ADL is known to underestimate the total lignin content, as it does not take into account the acid soluble lignin (Jung *et al.*, 1999). Both methods, however, seem in agreement on the extent of delignification by both fungi. CS showed a higher delignification capability on MS3 straw (87.6%), compared to MS1 (84.4%) and MS2 (82.4%). Similar observations were also recorded for LE-treated MS3 straw (80.1%), compared to MS1 and MS2 (~75%).

To explain this observation, we assessed the lignin structural features of all straws. The untreated MS3 straw contained 6% more syringyl (S) unit compounds, compared to the other straws. This also resulted in a higher S to guaiacyl (G) ratio in MS3 (0.49), compared to MS1 and MS2 straws (~0.45). Fungal pretreatment resulted in a minor changes of lignin subunit composition, with slight preference of fungi towards attacking the S units, which is in agreement with previous reports (Del Río *et al.*, 2002; Van Kuijk *et al.*, 2016). The results also indicates that both fungi can degrade all lignin subunits at the magnitudes of lignin removal. The S-units result in more linear structures and have a lower redox potential compared to the G-units (Del Río *et al.*, 2012; Vane *et al.*, 2003), which made them more susceptible to the fungal attack (Van Kuijk *et al.*, 2016). Hence, the relatively high S/G ratio may partly explain the higher fungal delignification of MS3 straw. Nevertheless, the S/G ratio was poorly correlated to the total amount of lignin (Pearson's  $r = -0.17$ ), due to a smaller change in the S/G ratio. Fungal pretreatment resulted in reduced amounts of vinyl products, mainly 4-Vinylguaicol (Table 7-S1). These compounds are also derived from ferulic acids that links lignin to its associated

**Table 7.2.** Characterization of lignin and its structural moieties in different straw maturities (MS), treated with *C. subvermispora* (CS) and *L. edodes* (LE) for 7 weeks, using quantitative <sup>13</sup>C-1S Py-GC/MS.

Parameter	MS1 <sup>†</sup>			MS2			MS3		
	Control	CS	LE	Control	CS	LE	Control	CS	LE
Lignin content (% w/w DM)	23.7	4.7	8.1	22.6	4.8	6.9	23.5	3.5	5.9
Amount of lignin (g per 100 g OM)	24.2	3.8	6.2	23.1	4.1	5.6	24.0	3.0	4.8
Lignin aromatic unit (%) <sup>*</sup>									
H	8.9	13.3	11.1	9.3	13.3	11.9	8.8	13.9	11.4
G	62.6	61.3	63.3	62.5	59.3	62.9	61.2	59.3	62.4
S	28.5	25.5	25.6	28.1	27.5	25.2	30.0	26.8	26.2
S/G	0.46	0.42	0.40	0.45	0.46	0.40	0.49	0.45	0.42
Structural moieties (%)									
Unsubstituted	4.8	11.4	7.7	4.7	12.2	9.3	5.2	13.7	10.0
Methyl	2.0	3.7	2.9	2.1	3.8	3.0	2.3	4.5	3.5
Ethyl	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2
Vinyl	29.9	24.8	28.4	30.5	23.9	27.3	30.4	20.3	26.9
C <sub>α</sub> -oxidized	3.2	20.1	8.4	3.4	21.3	10.2	3.2	26.1	10.6
C <sub>β</sub> -oxidized	1.6	3.7	2.3	1.5	3.7	2.6	1.6	3.9	2.7
C <sub>γ</sub> -oxidized	57.0	46.8	52.0	56.2	46.8	51.1	55.7	47.8	49.8
Miscellaneous	1.9	1.9	2.1	1.9	1.8	1.8	2.0	1.7	2.0

Values are averages of 3 technical replicates. No statistics were carried out on the data.

<sup>†</sup> MS: maturity stages around harvest with 2 weeks differences among MS1, MS2 and MS3.

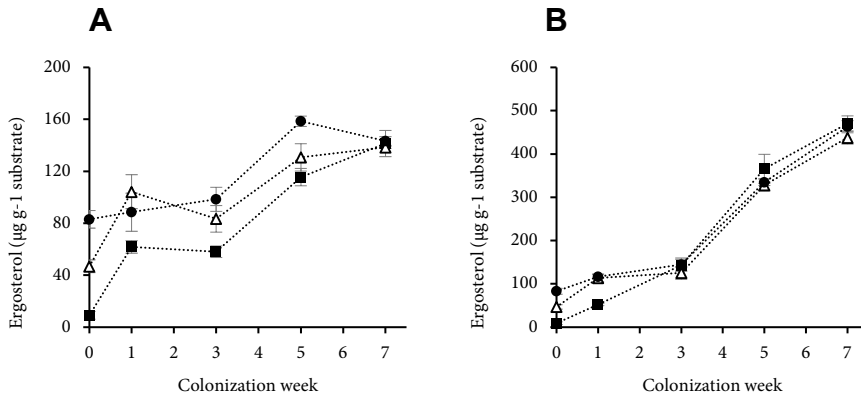
<sup>\*</sup> G: guaiacyl lignin subunit, H: *p*-hydroxyphenyl unit, S: syringyl unit.

carbohydrates (Buranov and Mazza, 2008). The reduction of vinyl products was more pronounced on the more mature straw, particularly for the CS treatment. Other notable changes including the increases of unsubstituted and C<sub>α</sub>-oxidized compounds with increasing straw maturity, particularly with the CS treatment, despite no difference was observed in the abundances of these compounds in the untreated straws. These observations clearly indicate more extensive degradation of inter-unit linkages within the lignin macromolecule of the more mature straw, which resulted in residual lignin with low intact linkages (Van Kuijk *et al.*, 2016; Zeng *et al.*, 2014). The ADL and Py-GC/MS assessments confirm the preference of both fungi in degrading the lignin of more mature straw – both by decreasing the amount of residual lignin and structural modifications.

### *Fungal growth*

A successful colonization of the substrate is an important prerequisite for an effective fungal pretreatment (Van Kuijk *et al.*, 2015b). Therefore, we assessed the growth of both fungi during the 7-week colonization period using an ergosterol assay (Fig. 7.1) (Mille-Lindblom *et al.*, 2004; Niemenmaa *et al.*, 2008). The baseline ergosterol content at week 0 was significantly ( $P < 0.001$ ) lower in the untreated MS1 straw and increased with increasing maturity. The higher ergosterol content in MS3 straw may be due to its high water potential (high osmotic gradient) which lead to a high activity and growth of field fungi (Magan and Lynch, 1986). Another possible explanation is post-harvest fungal growth, which is a common occurrence (Schmidt *et al.*, 2016). Although all processed straw was autoclaved prior to the fungal pretreatment, the persistence of ergosterol in the sterilized straw has been reported in several other studies (Mille-Lindblom *et al.*, 2004; Nayan *et al.*, 2017). The differences in the baseline ergosterol contents among the straw types produced unique colonization characteristics of both fungi, especially during the early growth stage. The growth of both fungi on MS3 at the beginning of the colonization weeks appeared slower, compared to their growth on MS1 and MS2. It can be that the existing ergosterol (or other components), belonging to the endogenous field fungi, may have ‘masked’ the initial growth of CS and LE. On MS3 straw, both fungi may have recycled these endogenous compounds and incorporated them in their own biomass, thus thereby erroneously indicating a slower growth or inability of these fungi to colonize the straw during early weeks. The dynamic in the fungal growth could be seen throughout the colonization period, especially for CS, which showed a low persistency in growth. This observation indicates a weaker colonization trait for this fungus on different straw types, compared to LE.





**Fig. 7.1.** Growth (based on ergosterol data) of *C. subvermisporea* (A) and *L. edodes* (B) on the wheat straw harvested at different maturity stages – MS1 (■), MS2 (△) and MS3 (●), for 7 weeks. Error bars indicate standard deviations.

#### *Assessing in vitro rumen degradability of the straw*

The effectiveness of both fungi to improve the rumen degradability of the straw was assessed by the *in vitro* gas production technique (IVGP), which has been used as a decisive parameter in selecting the strains used in the present study (Nayan *et al.*, 2018). Table 7.3 summarizes the IVGP and its kinetic parameters for straw of different maturities, treated with the two fungal species. The changes in IVGP has been well explained by the changes in cell wall composition of the substrate (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2017). Although the control straws were noticeably different in their cell wall compositions, the IVGPs of all straw maturities were not different. There was no effect of maturity stage on the IVGP of the fungal-treated wheat straws. CS and LE significantly ( $P < 0.05$ ) improved the IVGP of all straw types by 33.6 and 20.4%, respectively, which was within the range of previous reports (Nayan *et al.*, 2017, 2018). CS performed significantly ( $P < 0.05$ ) better than LE in improving the degradability of the straw at all stages. The highest increase in the IVGP by both fungal pretreatments was observed on MS1 straw with 38.4 and 23.3% increases for CS and LE, respectively.

The kinetic parameters were determined using a biphasic model approach (Groot *et al.*, 1996) to differentiate the IVGP profile of all straws. This approach has been used to differentiate the fermentability of two fungal-treated wheat straws with similar total IVGP (Nayan *et al.*, 2017). All fungal treated straws showed a better kinetic profile than control – among others, a shorter half time to the asymptotic gas production ( $B_2$ ) and a steeper

**Table 7.3.** *In vitro* gas production and its kinetic parameters for wheat straw of different maturity stages, treated with *C. subvermispota* (CS) and *L. edodes* (LE) for 7 weeks.

Maturity stage †	Treatment	IVGP	Kinetic parameters					
			$A_1$	$A_2$	$B_2$	$C_2$	$t_{Rm2}$	$R_{m2}$
MS1	Control	241.4 <sup>a</sup>	32.0 <sup>a</sup>	193.6 <sup>a</sup>	13.93 <sup>c</sup>	2.39 <sup>a</sup>	15.99 <sup>bc</sup>	0.087 <sup>a</sup>
	CS	334.2 <sup>de</sup>	61.4 <sup>b</sup>	262.8 <sup>de</sup>	10.52 <sup>a</sup>	2.88 <sup>cde</sup>	13.09 <sup>a</sup>	0.143 <sup>c</sup>
	LE	297.7 <sup>bc</sup>	52.3 <sup>b</sup>	231.5 <sup>b</sup>	12.72 <sup>b</sup>	2.74 <sup>b</sup>	15.57 <sup>b</sup>	0.112 <sup>b</sup>
MS2	Control	250.6 <sup>a</sup>	34.5 <sup>a</sup>	198.1 <sup>a</sup>	14.47 <sup>c</sup>	2.50 <sup>a</sup>	17.01 <sup>d</sup>	0.088 <sup>a</sup>
	CS	323.6 <sup>cde</sup>	61.5 <sup>b</sup>	253.2 <sup>cde</sup>	10.70 <sup>a</sup>	2.94 <sup>de</sup>	13.39 <sup>a</sup>	0.145 <sup>c</sup>
	LE	307.0 <sup>bcd</sup>	50.3 <sup>b</sup>	243.8 <sup>bc</sup>	13.15 <sup>b</sup>	2.79 <sup>bc</sup>	16.20 <sup>bcd</sup>	0.111 <sup>b</sup>
MS3	Control	254.5 <sup>a</sup>	34.5 <sup>a</sup>	202.3 <sup>a</sup>	14.36 <sup>c</sup>	2.48 <sup>a</sup>	16.84 <sup>cd</sup>	0.088 <sup>a</sup>
	CS	339.5 <sup>e</sup>	60.6 <sup>b</sup>	269.8 <sup>e</sup>	10.48 <sup>a</sup>	3.00 <sup>e</sup>	13.20 <sup>a</sup>	0.152 <sup>c</sup>
	LE	293.4 <sup>b</sup>	37.3 <sup>a</sup>	245.2 <sup>bcd</sup>	13.06 <sup>b</sup>	2.79 <sup>bc</sup>	16.09 <sup>bc</sup>	0.112 <sup>b</sup>
RMSE		17.00	7.87	10.98	0.39	0.07	0.52	0.005

Values with different superscripts within a column are significantly ( $P < 0.05$ ) different. RMSE: Root mean square error; IVGP: cumulative *in vitro* gas production after 72 h ( $\text{ml g}^{-1}$  OM);  $A_1$ ,  $A_2$ : asymptotic gas production ( $\text{ml g}^{-1}$  OM) phase 1 and 2, respectively;  $B_2$ : half time of the maximum gas production of phase 2 (h);  $C_2$ : parameters determine the curvature of the graph;  $t_{Rm2}$ : time of the maximum fractional rate of substrate degradation (h);  $R_{m2}$ : maximum fractional rate of substrate degradation ( $\text{h}^{-1}$ ).

† Maturity stages around harvest with 2 weeks differences among MS1, MS2 and MS3.

curve (greater  $C_2$ ). There was no effect of straw maturity on the kinetic parameters of the fungal-treated straws; although a numerical increase of fractional fermentation rate at phase 2 ( $R_{m2}$ ) could be seen for CS-treated straws with increasing maturity. This observation indicates a strong capability of both fungi in improving the degradability of these straws to a similar extent.

### Effect of different cultivars

#### *Mass balances for wheat straw of different cultivars*

Table 7.4 summarizes the mass balances of the different wheat straw cultivars, treated and not treated with fungi. There was considerable variation in the amount of nutrients among the different straw cultivars, particularly in the DM and ash contents. The absolute amounts of OM in the starting materials were comparable among the different untreated

**Table 7.4.** Mass balances for wheat straw of different cultivars, treated with *C. subvermispora* (CS) and *L. edodes* (LE) for 7 weeks.

Cultivar <sup>†</sup>	Treatment	DM (g kg <sup>-1</sup> )	Ash (g kg <sup>-1</sup> DM)	Amount (g per 100 g of starting OM) <sup>‡</sup>						
				OM	Cell	Hcell	ADL	Sugar	CP	NI
CV1	Control	173.9 <sup>ef</sup>	26.9 <sup>k</sup>	100.0 <sup>a</sup>	51.1 <sup>abc</sup>	32.9 <sup>a</sup>	6.5 <sup>b</sup>	0.5 <sup>h</sup>	1.8 <sup>fg</sup>	7.2 <sup>d</sup>
	CS	156.9 <sup>gh</sup>	35.0 <sup>hi</sup>	87.5 <sup>e</sup>	46.8 <sup>f</sup>	9.6 <sup>g</sup>	0.4 <sup>e</sup>	2.4 <sup>a</sup>	2.9 <sup>ab</sup>	25.5 <sup>a</sup>
	LE	154.2 <sup>h</sup>	33.0 <sup>i</sup>	88.0 <sup>e</sup>	49.2 <sup>de</sup>	12.2 <sup>f</sup>	1.3 <sup>d</sup>	2.3 <sup>ab</sup>	2.8 <sup>ab</sup>	20.3 <sup>bc</sup>
CV2	Control	193.5 <sup>ab</sup>	75.1 <sup>e</sup>	100.0 <sup>a</sup>	50.4 <sup>cde</sup>	33.8 <sup>a</sup>	6.3 <sup>b</sup>	0.5 <sup>h</sup>	1.5 <sup>gh</sup>	7.5 <sup>d</sup>
	CS	177.0 <sup>cdef</sup>	85.1 <sup>c</sup>	97.1 <sup>b</sup>	49.7 <sup>cde</sup>	19.9 <sup>b</sup>	3.0 <sup>c</sup>	1.8 <sup>c</sup>	2.2 <sup>cdef</sup>	20.6 <sup>bc</sup>
	LE	186.5 <sup>bde</sup>	78.9 <sup>d</sup>	96.2 <sup>b</sup>	52.5 <sup>ab</sup>	17.9 <sup>bcd</sup>	3.3 <sup>c</sup>	2.1 <sup>bcd</sup>	2.1 <sup>def</sup>	18.2 <sup>c</sup>
CV3	Control	203.1 <sup>a</sup>	84.7 <sup>c</sup>	100.0 <sup>a</sup>	50.9 <sup>bcd</sup>	33.4 <sup>a</sup>	6.1 <sup>b</sup>	0.4 <sup>h</sup>	1.2 <sup>h</sup>	7.9 <sup>d</sup>
	CS	189.0 <sup>abcd</sup>	95.2 <sup>a</sup>	96.6 <sup>b</sup>	50.5 <sup>bcd</sup>	19.3 <sup>bc</sup>	2.8 <sup>c</sup>	1.5 <sup>f</sup>	2.2 <sup>cdef</sup>	20.4 <sup>bc</sup>
	LE	190.4 <sup>abc</sup>	90.7 <sup>b</sup>	95.7 <sup>bc</sup>	52.5 <sup>ab</sup>	17.4 <sup>cd</sup>	3.3 <sup>c</sup>	2.1 <sup>cd</sup>	2.3 <sup>cde</sup>	18.3 <sup>c</sup>
CV4	Control	191.2 <sup>abc</sup>	30.7 <sup>j</sup>	100.0 <sup>a</sup>	49.6 <sup>cde</sup>	33.6 <sup>a</sup>	6.6 <sup>ab</sup>	0.7 <sup>g</sup>	1.9 <sup>ef</sup>	7.6 <sup>d</sup>
	CS	163.2 <sup>gh</sup>	38.5 <sup>f</sup>	96.4 <sup>b</sup>	50.1 <sup>cde</sup>	16.6 <sup>de</sup>	2.9 <sup>c</sup>	1.9 <sup>de</sup>	2.6 <sup>bc</sup>	22.2 <sup>b</sup>
	LE	175.3 <sup>def</sup>	35.8 <sup>gh</sup>	94.2 <sup>cd</sup>	52.8 <sup>a</sup>	14.7 <sup>e</sup>	2.8 <sup>c</sup>	2.4 <sup>a</sup>	2.4 <sup>cd</sup>	19.1 <sup>c</sup>
CV5	Control	192.2 <sup>ab</sup>	29.9 <sup>j</sup>	100.0 <sup>a</sup>	49.2 <sup>de</sup>	33.0 <sup>a</sup>	7.2 <sup>a</sup>	0.6 <sup>gh</sup>	2.2 <sup>cde</sup>	7.9 <sup>d</sup>
	CS	170.1 <sup>fg</sup>	37.3 <sup>fg</sup>	95.4 <sup>bc</sup>	48.3 <sup>ef</sup>	16.0 <sup>de</sup>	2.7 <sup>c</sup>	2.0 <sup>de</sup>	3.2 <sup>a</sup>	23.1 <sup>ab</sup>
	LE	171.0 <sup>fg</sup>	35.2 <sup>ghi</sup>	92.9 <sup>d</sup>	50.7 <sup>bcd</sup>	15.3 <sup>e</sup>	2.9 <sup>c</sup>	2.2 <sup>abc</sup>	2.8 <sup>ab</sup>	19.0 <sup>c</sup>
RMSE		9.02	1.36	1.11	1.11	1.08	0.34	0.11	0.24	1.54

Values with different superscripts within a column are significantly ( $P < 0.05$ ) different. DM: dry matter; OM: organic matter; Cell: cellulose; Hcell: hemicellulose; ADL: acid detergent lignin; Sugar: free reducing sugar from ethanol-extract of wheat straw; CP: crude protein ( $N \times 6.25$ ); NI: unaccounted OM. RMSE: Root mean square error.

<sup>†</sup> CV1: *Britannia*; CV2: *Cellule*; CV3: *Henriks*; CV4: *Residence*; CV5: *Tabasco*.

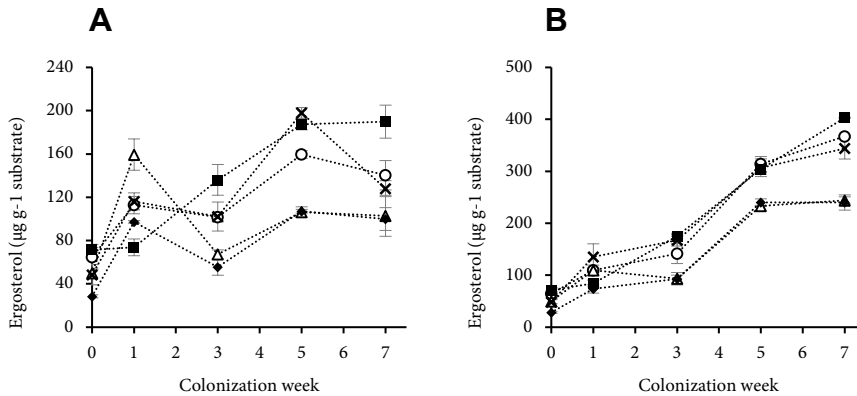
<sup>‡</sup> Calculated from the remaining materials for each treatment (g), using respective starting OM for each maturity stage.

straw cultivars (ranging from 36.3 to 37.6 g; Table 7.4). As in the previous experiment, the amount of nutrients was expressed per 100 g of starting OM. The starting amounts of all cell wall components were comparable among the different untreated straw cultivars, with CV5 high in lignin. Besides showing a high ash content, the untreated CV2 and CV3 straws contained lower amounts of CP, compared to the other straw cultivars.

There were significant ( $P < 0.05$ ) losses of OM in all fungal-treated straws, with relatively a higher loss observed for CV1 straw (~12.2%). The fungal degradation of lignin and hemicellulose for most straw cultivars were in the range of previous reports (Nayan *et al.*, 2017; Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015a). The fungal-treated CV1 straw, however, showed high losses of lignin (~86.5%) and hemicellulose (~67.0%). Previously, CS has been shown to be superior over LE in its delignification capability (Nayan *et al.*, 2018; Tuyen *et al.*, 2012). In the present study, the delignification capability of CS was surprisingly comparable to LE for most straw cultivars (56.2 vs 53.3%). CS degraded a significantly ( $P < 0.01$ ) higher amount of lignin in CV1, compared to LE (93.5 vs 79.6%). Significant losses of cellulose were observed for both fungi for the CV1 straw. While other fungal-treated straws showed slight changes in their cellulose contents, a significant increase of cellulose was observed for LE-treated CV2 (4%) and CV4 straws (6.5%). Similar observations were also reported in several other studies (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015a). The limitation in an accurate quantification of cellulose with regards to the interference of fungal biomass has been previously described (Nayan *et al.*, 2018). Nonetheless, the variable observation in cellulose content among studies (based on the gravimetric method) triggers an intriguing question on how these different types and batches of straw affect the fungal growth and the extent of utilization of the polysaccharides.

### *Fungal growth*

The growth of both fungi on wheat straw of different cultivars is illustrated in Fig. 7.2. The baseline ergosterol content varied considerably among all five cultivars, ranging from 28.4  $\mu\text{g g}^{-1}$  (CV3) to 71.4  $\mu\text{g g}^{-1}$  (CV5). LE showed a more consistent growth on all straw cultivars, compared to CS, although its overall growth was noticeably lower on CV2 and CV3 straw. Similar to the growth on MS3 in an earlier experiment (with high baseline ergosterol content), the growth of both fungi were more challenged on the CV5 straw. Due to a characteristically less formation of mycelium, CS was more affected by the variable baseline ergosterol content, compared to LE. On CV2 and CV3 straws, the CS



**Fig. 7.2.** Growth (based on ergosterol data) of *C. subvermispora* (A) and *L. edodes* (B) on the wheat straw of different cultivars – CV1 (O), CV2 (Δ), CV3 (◆), CV4 (×) and CV5 (■) for 7 weeks. Error bars indicate standard deviations.

growth appeared ‘stunted’ after a rapid increase in ergosterol at week 1, before its growth continued at a slower rate towards the end of the colonization weeks. There were also significant ( $P < 0.05$ ) decreases in the ergosterol content of CS grown on CV 1 and CV4 after week 5. These observations further indicate a weaker colonization trait of CS compared to LE on various types of straw used.

#### *Assessing in vitro rumen degradability of the straw*

The IVGP and its kinetic parameters for straw of different cultivars treated with CS and LE are summarized in Table 7.5. There were no differences in the IVGP between the different untreated straws, although a significantly ( $P < 0.05$ ) lower  $R_{m2}$  was observed for CV3 straw compared to CV1 and CV5. Although both fungi significantly ( $P < 0.001$ ) increased the IVGP of the straws (compared to controls), the magnitude of the effects were significantly ( $P < 0.001$ ) affected by the different straw cultivars. The IVGP of the fungal treated CV2 and CV3 were noticeably lower than the other treated straw cultivars with the difference between CS-treated CV3 and CV4 being significant ( $P < 0.05$ ). In addition, the  $R_{m2}$  of CV2 and CV3 treated with both fungi were significantly ( $P < 0.05$ ) lower than the other treated straw cultivars. Overall, CS treatment resulted in a significantly ( $P < 0.05$ ) higher increase in the IVGP, compared to LE (~43 vs. 29%).

**Table 7.5.** *In vitro* gas production and its kinetic parameters for wheat straw of different cultivars, treated with *C. subvermispora* (CS) and *L. edodes* (LE) for 7 weeks.

Cultivar †	Treatment	IVGP	Kinetic parameters					
			$A_1$	$A_2$	$B_2$	$C_2$	$t_{Rm2}$	$R_{m2}$
CV1	Control	223.8 <sup>a</sup>	16.9 <sup>b</sup>	199.9 <sup>a</sup>	14.45 <sup>de</sup>	2.41 <sup>a</sup>	16.68 <sup>bc</sup>	0.085 <sup>bcd</sup>
	CS	308.4 <sup>fg</sup>	49.4 <sup>g</sup>	255.6 <sup>ef</sup>	11.20 <sup>a</sup>	2.86 <sup>d</sup>	13.91 <sup>a</sup>	0.135 <sup>i</sup>
	LE	282.7 <sup>bcd</sup>	32.1 <sup>de</sup>	243.7 <sup>de</sup>	12.57 <sup>b</sup>	2.67 <sup>bcd</sup>	15.22 <sup>ab</sup>	0.110 <sup>h</sup>
CV2	Control	205.4 <sup>a</sup>	6.0 <sup>a</sup>	191.3 <sup>a</sup>	15.38 <sup>ef</sup>	2.41 <sup>a</sup>	17.76 <sup>cde</sup>	0.080 <sup>abc</sup>
	CS	294.6 <sup>defg</sup>	41.9 <sup>efg</sup>	245.0 <sup>de</sup>	13.31 <sup>bcd</sup>	2.55 <sup>abc</sup>	15.79 <sup>bc</sup>	0.099 <sup>efg</sup>
	LE	269.4 <sup>bc</sup>	33.5 <sup>de</sup>	224.6 <sup>bc</sup>	14.39 <sup>de</sup>	2.53 <sup>abc</sup>	17.01 <sup>bc</sup>	0.090 <sup>de</sup>
CV3	Control	203.9 <sup>a</sup>	9.2 <sup>ab</sup>	183.5 <sup>a</sup>	16.96 <sup>g</sup>	2.44 <sup>a</sup>	19.68 <sup>e</sup>	0.073 <sup>a</sup>
	CS	289.2 <sup>cdef</sup>	36.7 <sup>def</sup>	240.7 <sup>cde</sup>	14.14 <sup>cde</sup>	2.51 <sup>abc</sup>	16.63 <sup>bc</sup>	0.091 <sup>def</sup>
	LE	260.7 <sup>b</sup>	27.5 <sup>cd</sup>	219.6 <sup>b</sup>	14.81 <sup>e</sup>	2.49 <sup>abc</sup>	17.37 <sup>cd</sup>	0.086 <sup>cd</sup>
CV4	Control	213.1 <sup>a</sup>	9.4 <sup>ab</sup>	192.8 <sup>a</sup>	16.28 <sup>fg</sup>	2.47 <sup>ab</sup>	19.04 <sup>de</sup>	0.077 <sup>ab</sup>
	CS	314.4 <sup>g</sup>	37.9 <sup>def</sup>	266.9 <sup>f</sup>	12.86 <sup>bc</sup>	2.73 <sup>cd</sup>	15.69 <sup>abc</sup>	0.111 <sup>h</sup>
	LE	281.0 <sup>bcd</sup>	30.4 <sup>d</sup>	241.1 <sup>cde</sup>	13.40 <sup>bcd</sup>	2.59 <sup>abc</sup>	16.04 <sup>bc</sup>	0.099 <sup>fg</sup>
CV5	Control	209.9 <sup>a</sup>	18.2 <sup>bc</sup>	183.1 <sup>a</sup>	15.12 <sup>ef</sup>	2.44 <sup>ab</sup>	17.56 <sup>cd</sup>	0.082 <sup>bcd</sup>
	CS	305.2 <sup>efg</sup>	47.4 <sup>g</sup>	252.9 <sup>ef</sup>	12.23 <sup>ab</sup>	2.89 <sup>d</sup>	15.24 <sup>ab</sup>	0.124 <sup>i</sup>
	LE	269.6 <sup>bcd</sup>	34.9 <sup>de</sup>	225.8 <sup>bc</sup>	12.58 <sup>b</sup>	2.62 <sup>abc</sup>	15.12 <sup>ab</sup>	0.107 <sup>gh</sup>
RMSE		13.38	6.00	10.28	0.71	0.13	1.07	0.005

Values with different superscripts within a column are significantly ( $P < 0.05$ ) different. RMSE: Root mean square error; IVGP: cumulative *in vitro* gas production at 72 h ( $\text{ml g}^{-1}$  OM);  $A_1$ ,  $A_2$ : asymptotic gas production ( $\text{ml g}^{-1}$  OM) phase 1 and 2, respectively;  $B_2$ : half time of the maximum gas production of phase 2 (h);  $C_2$ : parameters determine the curvature of the graph;  $t_{Rm2}$ : time of the maximum fractional rate of substrate degradation (h);  $R_{m2}$ : maximum fractional rate of substrate degradation ( $\text{h}^{-1}$ ).

† CV1: *Britannia*; CV2: *Cellule*; CV3: *Henrik*; CV4: *Residence*; CV5: *Tabasco*.

Correlating the IVGP to the fungal growth (ergosterol) is rather complex, although its relationship was statistically significant ( $r = 0.56$ ;  $P < 0.001$ ). CS, which possessed a weaker colonization trait on any straw type, resulted in a more degradable straw compared to LE. Hence, the growth ‘inconsistency’ seen in CS more likely suggests a higher dynamic nutrient utilization and recycling of the field fungi biomass, contributing to an apparent slow growth rate. It does not indicate the inability of this particular fungus to successfully colonize the different straw types. When scaling-up the bioprocess in practice, the slow growth of CS may lead to a problem with undesirable microbial growth on the substrate. Nevertheless, an improved and optimized inoculation method can be

used to ensure a quick colonization of the substrate. In both trials above, these high potential strains showed a high persistency in improving the degradability of the straw, although different straw cultivars affected the IVGP of the CS and LE treatments. Significant ( $P < 0.05$ ) correlations were found between the IVGP and the cell wall compositions in both trials, with stronger correlations to lignin ( $r \sim -0.91$ ) and hemicellulose ( $r \sim -0.92$ ), than to cellulose ( $r \sim 0.51$ ). Nonetheless, the current results indicate that a careful assessment has to be made when describing the chemical changes (using the unspecific gravimetric method) and relate them to the subsequent changes in IVGP. As aforementioned, the interference of fungal biomass has to be taken into account. Based on this consideration, the IVGP is, therefore, the most potent method in assessing the success and effectiveness of a particular fungus in improving the ruminal degradability of different straw types.

## CONCLUSION

Different straw types influenced the characteristics of *C. subvermispota* and *L. edodes* in degrading lignin. A more pronounced degradation of lignin was observed on mature straw, which was further confirmed by quantitative  $^{13}\text{C}$ -IS Py-GC/MS of the wheat straw. Variable results of lignin degradation were observed with different straw cultivars. Both high potential strains of *C. subvermispota* and *L. edodes* were able to improve the ruminal degradability of wheat straw, regardless of various straw types (maturity and cultivar) investigated. The magnitude of the effect, however, was only affected by different straw cultivars, but not by different maturities around harvest. Under all circumstances, *C. subvermispota* was more capable than *L. edodes* in improving the degradability of wheat straw. *L. edodes* had a better adaption in colonizing different straw types compared to *C. subvermispota*.

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Supplementary information for:

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## **Improving ruminal digestibility of various wheat straw types by white-rot fungi**

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**Table 7-S1.** Identities and relative abundances of lignin-derived compounds in different straw maturities (MS), treated with *C. subvermispora* (CS) and *L. edodes* (LE) for 7 weeks

Label	Compound	Origin	Ret. Time	MW <sup>12</sup> C	Structure*	MS1			MS2			MS3		
						Control	CS	LE	Control	CS	LE	Control	CS	LE
1	Phenol	H	10	94	Unsub	0.7	3.4	1.9	0.8	3.5	2.5	0.7	4.4	2.5
2	Guaiacol	G	10.29	124	Unsub	2.2	5.2	3.4	2.2	5.6	4.2	2.4	6.2	4.6
3	2-Methylphenol	H	11.25	108	Methyl	0.2	0.7	0.5	0.2	0.9	0.6	0.2	1.0	0.7
4	4-Methylphenol	H	12.19	108	Methyl	0.4	1.3	0.8	0.4	1.3	1.0	0.4	1.8	1.0
5	4-Methylguaiacol	G	12.97	138	Methyl	0.8	1.0	1.0	0.8	0.9	0.9	0.9	1.0	1.0
6	2,4-Dimethylphenol	H	13.41	122	Methyl	0.1	0.2	0.1	0.1	0.3	0.2	0.1	0.4	0.2
7	4-Ethylguaiacol	G	15.14	152	Ethyl	0.1	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2
8	4-Vinylguaiacol	G/FA	16.59	150	Vinyl	19.7	15.7	18.3	19.8	15.1	17.5	20.0	12.8	17.7
9	4-Vinylphenol	H/PCA	16.7	120	Vinyl	7.3	7.1	7.5	7.8	6.8	7.4	7.3	5.9	6.7
10	Eugenol	G	17.2	164	Misc	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
11	Syringol	S	17.94	154	Unsub	1.9	2.8	2.3	1.8	3.1	2.6	2.1	3.1	2.9
12	<i>cis</i> -Isoeugenol	G	18.57	164	Misc	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1
13	<i>trans</i> -Isoeugenol	G	19.83	164	Misc	0.8	0.9	1.0	0.8	0.8	0.8	0.8	0.8	0.9
14	4-Methylsyringol	S	20.18	168	Methyl	0.6	0.5	0.5	0.6	0.4	0.4	0.7	0.4	0.5
15	Vanillin	G	20.27	152	C <sub>α</sub> -ox	1.0	2.9	1.6	1.0	2.8	1.7	0.9	3.0	1.7
16	Homovanillin	G	21.73	166	C <sub>β</sub> -ox	0.5	1.0	0.7	0.5	1.0	0.7	0.5	1.0	0.7
17	Acetovanillone	G	22.19	166	C <sub>α</sub> -ox	0.2	1.4	0.7	0.3	1.4	0.8	0.2	1.7	0.9
18	4-Hydroxybenzaldehyde	H	22.92	122	C <sub>α</sub> -ox	0.1	0.5	0.3	0.1	0.5	0.3	0.1	0.5	0.2
19	4-Vinylsyringol	S	23.24	180	Vinyl	2.9	1.9	2.6	2.9	2.0	2.4	3.1	1.7	2.5
20	Guaiacylacetone	G	23.41	180	C <sub>β</sub> -ox	0.3	1.1	0.6	0.3	1.1	0.7	0.3	1.1	0.7
21	Guaiacyl vinyl ketone	G	24.36	178	C <sub>α</sub> -ox	0.1	0.3	0.2	0.1	0.3	0.2	0.1	0.4	0.2
22	Vanilloyl acetaldehyde	G	24.6	194	C <sub>α</sub> -ox, C <sub>β</sub> -ox	0.2	7.7	2.2	0.2	7.9	3.3	0.3	10.8	3.2

Table 7-S1. (*continued*)

23	<i>trans</i> -4-Propenylsyringol	S	26.08	194	Misc	0.9	0.6	0.8	0.8	0.7	1.0	0.5	0.7
24	Syringaldehyde	S	26.65	182	C <sub>α</sub> -ox	0.7	1.1	0.9	0.8	1.2	0.8	1.2	0.9
25	<i>cis</i> -Coniferyl alcohol	G	26.72	180	C <sub>γ</sub> -ox	1.2	0.9	1.2	1.1	0.8	1.1	0.7	1.0
26	Homosyringaldehyde	S	27.64	196	C <sub>α</sub> -ox	0.3	0.5	0.4	0.3	0.5	0.4	0.6	0.4
27	Acetosyringone	S	28.09	196	C <sub>α</sub> -ox	0.6	1.2	0.9	0.6	1.3	0.9	1.3	1.0
28	<i>trans</i> -Coniferyl alcohol	G	28.43	180	C <sub>γ</sub> -ox	33.1	20.6	29.9	32.9	19.0	28.3	17.1	26.7
29	<i>trans</i> -Coniferaldehyde	G	28.8	178	C <sub>γ</sub> -ox	2.0	2.1	2.2	2.1	2.0	2.3	2.2	2.5
30	Syringylacetone	S	29.02	210	C <sub>α</sub> -ox	0.5	1.2	0.7	0.4	1.2	0.7	1.2	0.8
31	Syringoyl acetaldehyde	S	29.78	224	C <sub>α</sub> -ox, C <sub>γ</sub> -ox	0.2	5.0	1.7	0.3	5.9	2.1	0.2	7.3
32	<i>cis</i> -Sinapyl alcohol	S	31.95	210	C <sub>γ</sub> -ox	1.1	0.6	0.8	1.0	1.0	0.8	1.1	0.9
33	<i>trans</i> -Sinapyl alcohol	S	33.66	210	C <sub>γ</sub> -ox	17.2	7.9	11.9	16.9	7.7	10.8	6.9	10.7
34	<i>trans</i> -Sinapaldehyde	S	33.88	208	C <sub>γ</sub> -ox	1.9	2.1	2.1	1.7	2.6	2.4	1.8	2.4

G: guaiacyl lignin unit; H: p-hydroxyphenyl unit; S: syringyl unit; FA: ferulic acid; PCA: *p*-coumaric acid; Ret. Time: Retention time; MW <sup>12</sup>C: Molecular weight

\* Unsub: unsubstituted; Methyl, ethyl, vinyl: substituted compounds; Misc: miscellaneous structures; C<sub>α</sub>-ox, C<sub>γ</sub>-ox: compounds with oxidation at C<sub>α</sub>, C<sub>γ</sub> position, respectively.



“

Be kind, for whenever kindness becomes part of something, it beautifies it. Whenever it is taken from something, it leaves it tarnished.

”

- MUHAMMAD ﷺ

# General discussion





### Understanding fungal variations

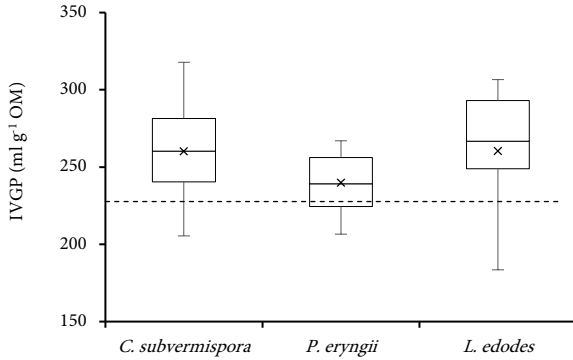
The main focus of this thesis is to discern biological variation among different strains/species and select the best performing fungi as candidates for the bioprocessing of wheat straw into ruminant feed. In addition, the capabilities of the selected fungi in improving the degradability of various straw types were also studied. The selected high potential fungi were assessed for their capabilities to solubilize protein, modify lignin structures and improve various types of wheat straw. In Chapter 2, only two different strains of *C. subvermispota* were assessed to better understand variation that may exist between the two genetically related fungi. A qualitative assessment of 3 main lignin-degrading enzymes – lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase, was performed in an attempt to relate the activity of these enzymes to the changes observed in other measured variables, in particular the lignin (ADL) content, and to see if this could be a potential selection parameter for Chapter 3. The results, however, showed no correlation of enzyme activities to the changes in the ADL content over the 7-week period, which is in agreement with previous reports (Sharma and Arora, 2010; Shrivastava *et al.*, 2011). This emphasizes the limited knowledge we have on the mechanism involved in the degradation of lignocellulose by these fungi and, for the time being, the inability to use enzyme assays in selecting the right fungi for the purpose in Chapter 3. A secretome analysis could be an answer for an in-depth understanding of variation in the enzyme expressions by different fungal species (Bianco and Perrotta, 2015). Other variables that are not measured, but might be significant to the understanding of fungal variations, include production of organic acids and secondary metabolites. In general, three basic parameters were used in the main experimental chapters (Chapter 2, 3 and 7) to assess the performance of each fungus tested: (1) the fermentation in rumen fluid, using the *in vitro* gas production (IVGP) technique, (2) assessment of fungal growth using ergosterol assay and (3) mass balance.

### Gas production as a screening criterion

In Chapter 2, a distinct characteristic of two genetically related fungi was revealed, particularly in their growth and the kinetics of IVGP, although there was only a slight difference in the total IVGP of both fungal-treated wheat straws after 7 weeks. The assessment of IVGP is important as a tool of research in this thesis, since this is a good indicator for the fermentability in the rumen of ruminants (Cone *et al.*, 1996). Therefore, the IVGP was used as a decisive screening parameter for the best performing fungi in Chapter 3. In the literature, several studies carried out fungal screening based on their

enzyme activities (Chang *et al.*, 2012; Cruz Ramírez *et al.*, 2012), and some studies assessed the changes in the lignin and carbohydrate contents as criteria to screen for selective fungi (Blanchette, 1984; Li *et al.*, 2008; Tripathi *et al.*, 2008). While the inconvenience of enzyme assays has been described earlier, the assessment of the changes in the cell wall compositions and other nutrients using the standard laboratory analyses are reliable methods to examine the selectivity of fungi. However, the data obtained in Chapter 3 and 7 show that the changes in cell wall contents as determined by the routine method (Van Soest *et al.*, 1991), does not always explain the subsequent effect on the gas production. For instance, strain CS12 degraded 17.6% of the ADL content, while strain PE2 caused a significantly higher loss of ADL (23.5%). However, a significantly higher IVGP was observed for CS12-treated wheat straw compared to PE2 (291.5 vs. 257.9 ml g<sup>-1</sup> OM) (Chapter 3). In Chapter 7, the ADL content of the untreated straw increased with increasing maturity; their IVGPs, however, were not significantly different. In addition, the ADL method underestimates the total lignin content due to the loss of acid-soluble lignin (Jung *et al.*, 1999), which is definitely a major shortcoming in quantifying the true extent of delignification by a particular fungus. As discussed in Chapter 3, 5 and 7, one of the limitations of using the Van Soest *et al.* (1991) method to quantify the cell wall components, particularly cellulose, is the inclusion of chitin and  $\beta$ -glucan from fungal biomass in the acid detergent fraction, which will be discussed in a later section. The structural similarities of fungal biomass with the substrate polymers contribute to inaccuracy in mass balance. These observations suggest the insufficiency of this routine method in assessing the effectiveness of a particular fungus. The relevance of using the Van Soest method in explaining the subsequent effect on IVGP has been discussed in a greater depth in Chapter 5. Based on these premises, the IVGP is, therefore, a robust and effective technique in selecting the best performing fungal strain/species.

Assessment of the IVGP showed a clear variation in the capabilities of different strains within a particular fungal species, as illustrated in Fig. 8.1. The IVGP values (for all individual observations) ranged from 205.5 to 317.8 ml g<sup>-1</sup> OM for *C. subvermispora* strains; while the ranges of IVGP for *P. eryngii* and *L. edodes* strains were 206.6 to 267.0 and 183.5 to 306.6 ml g<sup>-1</sup> OM, respectively. The mean IVGP of the untreated straw was 227.7  $\pm$  0.5 ml g<sup>-1</sup> OM. This shows that not all strains of these previously identified high potential fungal species (Tuyen *et al.*, 2012) can result in a significant improvement of the nutritive value of wheat straw. The boxplot (Fig. 8.1) also shows a larger variation among *C. subvermispora* and *L. edodes* strains compared to *P. eryngii*. Nonetheless, a higher



**Fig. 8.1.** Boxplot showing the variation in *in vitro* gas production (IVGP) of wheat straw treated with different strains for each fungal species after 7 weeks. Mean values are indicated with 'x'. Dashed-line indicates the mean for IVGP of the untreated wheat straw (227.7 ml g<sup>-1</sup> OM). The whiskers indicate the variability outside the upper and lower quartiles.

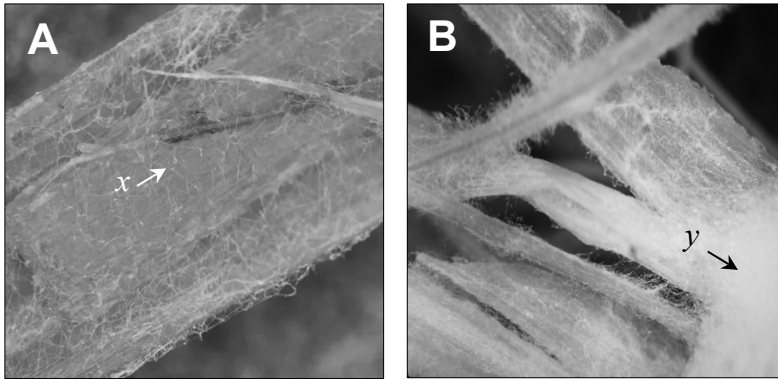
number of *L. edodes* strains were able to improve the straw degradability (median = 266.7 ml g<sup>-1</sup> OM), followed by *C. subvermispورا* and *P. eryngii* with a median of 260.3 and 239.3 ml g<sup>-1</sup> OM, respectively. Three strains for each species with a high potential in improving the ruminal degradability of wheat straw were selected in Chapter 3. They were (in order of IVGP for each species): CS1, CS6 and CS12 (*C. subvermispورا*); PE6, PE2 and PE3 (*P. eryngii*); LE8, LE7 and LE10 (*L. edodes*). The selection carried out in Chapter 3 was based on the maximum IVGP reached after 7 weeks of treatment; its changes over time, however, have not been discussed or considered in the selection although the measurement for the weekly samples were also done. There are two reasons to justify this decision. First, the changes in IVGP over time for all treatments followed a similar pattern as previously reported in Chapter 2 and the literature (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015), where a decrease in IVGP was observed at the beginning of the colonization weeks. In general, all *C. subvermispورا* and *L. edodes* strains caused a decrease in the IVGP in the first week, before increasing until the end of the treatment period; while decreases of up to 3 week were observed for *P. eryngii* strains. Secondly, none of the IVGP values of the earlier weeks was significantly higher than the IVGP at week 7, except a numerical decrease was observed for LE6-treated straw (286.6 to 283.2 ml g<sup>-1</sup> OM). Meanwhile, only wheat straw treated with LE4 and LE5 showed a constant decrease in the IVGP from the beginning of the colonization week. The two fungi were not capable of colonizing the wheat straw. These considerations support the reliability of the assessment (and

screening) of fungi based on the IVGP after 7 weeks of treatment in Chapter 3. Nonetheless, selection based on the time of improvement in IVGP is undeniably important, especially when it comes to implementing this pretreatment method at an industrial scale, where efficiency in time matters. This selection perspective, together with other possible interventions to accelerate fungal pretreatment, can be considered for future research.

### **Fungal growth and the difficulties in mass balances**

The ergosterol assay has been used to estimate fungal growth in Chapter 2, 3 and 7. With proper storage and care, the ergosterol in the untreated straw, which may originate from dead field fungi, are persistent even after autoclaving, although it can be subjected to photochemical degradation (Mille-Lindblom *et al.*, 2004). There were considerable variations in the mean ergosterol contents measured for the untreated straw in Chapter 2, 3 and 7, which ranged from 9.2 to 83.1  $\mu\text{g g}^{-1}$  substrate ( $n = 42$ ). In Chapter 7, a high ergosterol content was found for the mature straw, showing an accumulation of the field fungi. In the same batch of wheat straw (Chapter 3), fungi clearly varied in their growth, although all strains within each species showed comparable growth patterns. Different batches of straw seemingly affected the fungal growth. This was especially evident when CS1 and LE8 grew on the different straws collected around harvest (Chapter 7). In that chapter, both fungi showed a slower growth rate on a mature straw (MS3) at the beginning of the colonization, compared to when they were grown on less mature wheat straw (MS1 and MS2). The apparent slow initial growth of these fungi on MS3, is attributed to the recycling of endogenous fungi components, which ‘masked’ the actual growth. The variable growth patterns observed in the three experimental chapters show the dynamic of fungi upon colonizing various types and conditions of straw, which led to the problem of defining a successful colonization.

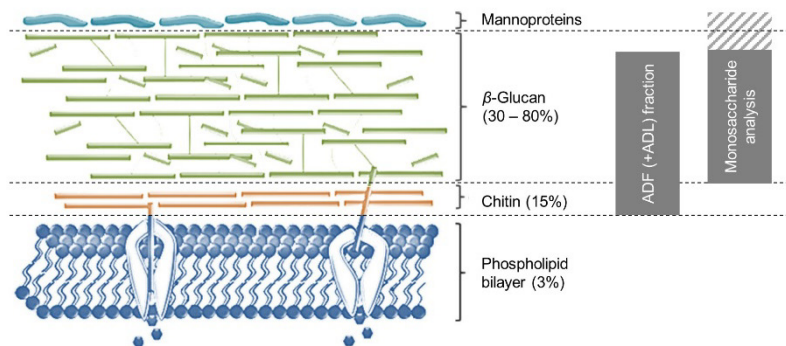
The rate of initial growth is also important in defining a successful substrate colonization by fungi. *C. subvermispora*, in particular CS1, possesses unique growth characteristics, where it produces a visible thin mycelium throughout the colonization weeks (Fig. 8.2), while being the most potential fungus in our collection for degrading lignin and improving the IVGP of straw. This fungus has a faster colonization compared to other species, as shown in Chapter 5, where a shorter period was required to harvest a fully colonized agar plate. Similar growth patterns were also observed when grown on wheat straw, with a steeper growing curve during the first week of colonization (Chapter



**Fig. 8.2.** Colonization of wheat straw by *C. subvermispورا* after 2 weeks (A) and 7 weeks (B) as viewed with a light microscope at 50 and 32 $\times$  magnification. In 2 weeks, a network of thin mycelia (x) already colonized most of the substrate surface, while a formation of thick aerial mycelia (y) can be seen after 7 weeks.

2, 3 and 7). *L. edodes* and *P. eryngii* strains clearly showed a longer lag phase, although both fungi generally produced thicker mycelia at week 7. A ‘stunted’ growth of *C. subvermispورا* on CV2 and CV3 after week 1 observed in Chapter 7 does not indicate the inability of this particular fungus to colonize those substrates. A decrease in ergosterol content was also observed for *C. subvermispورا* grown on MS3 and CV4 (Chapter 7), and also for all *P. eryngii* strains (Chapter 3) towards the end of the colonization. These observations indicate the dynamic of these fungi in utilizing available nutrients, as well as possible recycling of their own mycelia.

As heterotrophs, fungi hydrolyze polysaccharides for their growth and metabolism (Carlile *et al.*, 2001). These polysaccharides are partly incorporated into fungal biomass, or can be mineralized to CO<sub>2</sub> and water, explaining the losses in organic matter after fungal pretreatments. These changes present a great challenge, particularly for an accurate quantification of losses in cellulose, hemicellulose and lignin. As aforementioned, the inclusion of chitin and  $\beta$ -glucan from fungal biomass complicates the interpretation of mass balances, in relation to the efficiency of a particular fungus to depolymerize the cell wall polymers of the substrate. Fig. 8.3 illustrates a hypothetical proposition on the inclusion of fungal cell wall components in the measurements of cellulose, hemicellulose and lignin. Chitin is a polymer of  $\beta$ -(1 $\rightarrow$ 4)-linked *N*-acetyl-D-glucosamine (GlcNAc), which has a structural resemblance to cellulose ( $\beta$ -(1 $\rightarrow$ 4)-linked D-glucose units) and is



**Fig. 8.3.** Main components of the fungal cell wall and cell membranes, with estimated compositions (as a percentage of the total fungal biomass). A hypothetical proposition on the inclusion of these fungal components in acid detergent (ADF and ADL) and monosaccharide analysis is shown. The diagram is adapted from Fesel and Zuccaro (2016).

one of the most insoluble compounds in nature (Fesel and Zuccaro, 2016). Chitin is included in the ADF fraction, and probably in the ADL fraction as well due to the fact that a complete hydrolysis of chitin polymers requires a stronger concentrated acid (Einbu and Vårum, 2008). The acid hydrolysis step in the monosaccharide analysis (Englyst and Cummings, 1984) may also degrade part of the  $\beta$ -glucan layer of the fungal cell wall. Hence, it interferes with the quantification of cellulose and hemicellulose. Therefore, it was concluded in Chapter 5 that neither acid detergent nor monosaccharide analysis can produce an accurate quantification of the polysaccharides originating from the substrate. However, an accurate amount of lignin can be quantified using Py-GC/MS (Chapter 5) without interference of fungal biomass due the fact that lignification is an exclusive process occurring in higher plants (Ros Barceló, 1997). These observations provide a further justification of using IVGP as the main parameter in assessing the effectiveness of fungi in improving the nutritive value of wheat straw, and biomass in general.

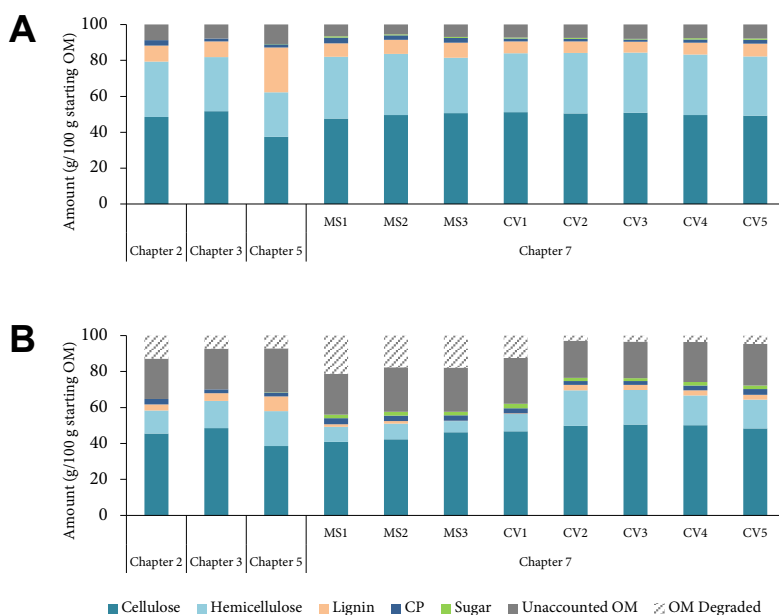
The question remains – can we accurately quantify the absolute amount of fungal biomass grown on wheat straw? It is almost impossible to segregate and quantify the fungal biomass grown on a solid substrate, such as wheat straw. Fungi penetrate deep into the cell wall matrix, even for a selective fungus, such as *C. subvermispota*, as revealed by light microscopy of the sections of fungal-treated straw (Van Kuijk *et al.*, 2017), which makes its absolute mass quantification difficult. Ergosterol, a sterol components in fungi,

constitutes about 0.1 to 2.4% of the dry mycelia mass with large variations among different fungal species (Klamer and Bååth, 2004). Correlating mass conversion from ergosterol obtained for fungi grown on a liquid medium to a solid substrate has been shown to be problematic, especially in comparing the biomass of different fungal species (Niemenmaa *et al.*, 2008). The ergosterol content varied with different media used and its content per dry weight of fungal mycelium is not constant at different stages of growth (Gao *et al.*, 1993). While it is a good qualitative estimate of fungal growth, quantitation of the total amount of fungal biomass on wheat straw, based on an estimated ergosterol conversion factor, may not solve the problem encountered with the mass balances. As reported in Chapter 5, the fungal biomass only accounted for less than 3% of the total OM. It can be inferred that, about 3% of the total cell wall contents – measured using the Van Soest *et al.* (1991) method, is associated with the fungal cell wall. The interference of fungal biomass in the total cell wall contents also complicate the understanding of the subsequent effects on IVGP. In Chapter 5, results demonstrated the fermentation of pure fungal biomass in the rumen and contribute to the total IVGP. The relationship between IVGP and the cell wall contents was also validated using more specific analyses of lignin and carbohydrates in Chapter 5. This is important since the ADL does not account for the acid-soluble lignin fraction, besides an interference of fungal biomass in cellulose contents. The specific analysis of lignin and carbohydrates were carried out using Py-GC/MS and monosaccharide analysis, respectively. In brief, inclusion of all lignin-derived compounds in the Py.lignin content led to a weaker inverse relationship with IVGP, compared to ADL. This indicates a slight degradation of the modified or fragmented lignin (with may contain intact associated carbohydrates) in the rumen. As for cellulose (and glucan) contents, neither of the two methods produce accurate quantification of the cellulose. The acid hydrolysis step in the monosaccharide analysis may also hydrolyze the  $\beta$ -glucan originating from the fungal cell wall (Fig. 8.3).

For mass balances of the (untreated) wheat straw, the percentage of recoveries for all quantified nutrients from the total amount of OM were comparable in Chapter 2, 3, 5 and 7 (ranging from 89.2 to 94.5%). The unaccounted OM (5.5 to 10.8%) may contain other nutrients, which were not measured in this thesis such as fat (~1.5%) (Gado *et al.*, 2017) and probably other water-soluble carbohydrates and nitrogen-containing compounds (Jurak *et al.*, 2015). After 7 weeks of fungal pretreatment, the recovery of OM significantly dropped and greatly varied among different fungal strains (69.9 to 88.3%); hence, increases in the percentage of unaccounted OM were observed (11.3 to 25.5%). This observation indicates a clear increase of the water-soluble nutrient fraction in fungal-

treated straw. Although an attempt to quantify these soluble lignin and carbohydrate fractions was made in Chapter 5, and soluble sugars in Chapter 7; none of these efforts were able to quantitate all unaccounted OM.

CS1 was consistently studied throughout this thesis (Chapter 2 to 7); hence, a good example to exhibit variations of the fungal pretreatment on the mass balances of different batches and types of straw, under the same treatment conditions. Figure 8.4 illustrates the mass balances of the control and CS1-treated wheat straw from different experimental chapters. The absolute amounts were expressed per 100 g of respective starting OM to compare across different chapters. For the untreated wheat straw, there were comparable amounts of cellulose (47.7 to 51.7 g/100 g OM) and hemicellulose (30.1 to 34.4 g/100 g OM) in Chapter 2, 3 and 7. Meanwhile, lignin was slightly variable (6.1 to 9.1 g/100 g OM). In chapter 7, the free sugars were also determined, which ranged from 0.4 to 0.7



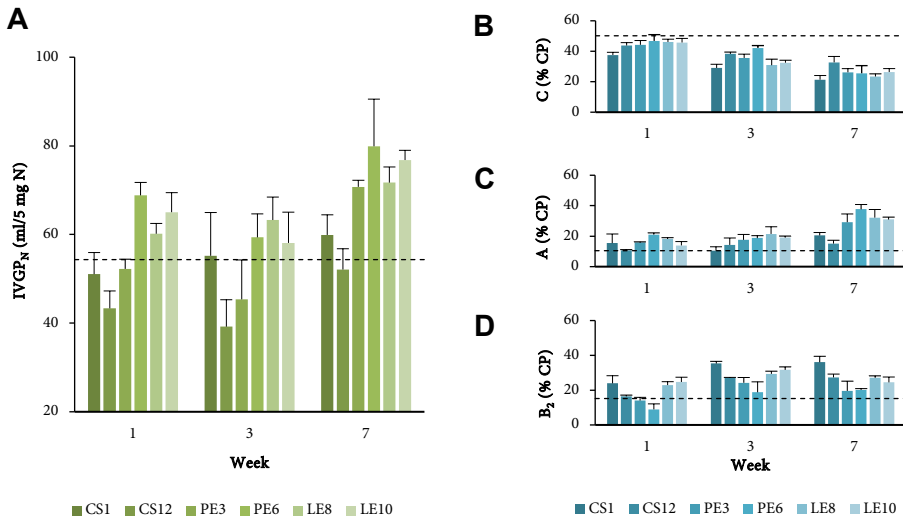
**Fig. 8.4.** Overview of the mass balances of the untreated wheat straw (A) and straw treated with *C. subvermisporea* strain CS1 for 7 weeks (B) in different chapters. The cell wall components in Chapter 2, 3 and 7 were measured using Van Soest *et al.* (1991). Free sugars were also determined in Chapter 7. In Chapter 5, lignin was quantified using Py-GC/MS, while cellulose and hemicellulose were quantified from the respective sugar polymers. All amounts were expressed as per 100 g of the respective starting organic matter (OM). MS1-3: different maturity stages of straw; CV1-5: different straw cultivars (*see* Chapter 7).



g/100 g OM for all different straw types. Using different analyses in Chapter 5, a higher amount of Py.lignin was quantified ( $25.1 \pm 0.9$  g/100 g OM); while lower amounts of cellulose (measured as glucan;  $37.4 \pm 0.7$  g/100 g OM) and hemicellulose (glucuron-arabinoxylan;  $24.8 \pm 0.5$  g/100 g OM) were observed, compared to the other chapters. Across different chapters, the amount of crude protein (CP) was variable (1.2 to 3.3 g/100 g OM). After 7 weeks of treatment, CS1 caused a variable effect on the degradation of total OM. Large losses of OM (12.5 to 21.3%) were observed for the treated straw in Chapter 2 and Chapter 7 (MS1–3, CV1). Most of CS1-treated straw had 0.8 to 14.6% loss of the amount of cellulose; in Chapter 5 and 7 (CV4), however, slight increases of cellulose were observed. The degradation of lignin and hemicellulose were mostly proportional with means of 69.2 and 56.0%, respectively, which conforms to various reports (Tuyen *et al.*, 2012; Van Kuijk *et al.*, 2015). CS1 showed a higher delignification capability compared to the other fungi. Nonetheless, its effect on various straw batches in this thesis was clearly variable (52.2 to 98.2%).

### Solubilization of protein

Two high-ranking strains of *C. subvermispora* (CS1 and CS12), *P. eryngii* (PE3 and PE6), and *L. edodes* (LE8 and LE10), were studied for their variation in the solubilization of protein (Chapter 4) and structural modification of wheat straw lignin (Chapter 6). The same set of fungi was also used in Chapter 5, to compare the two procedures of assessing lignin and carbohydrate contents, in relations to the IVGP. Throughout the thesis, most of the fungi tested, increased the concentration of CP in the substrate. Nonetheless, the magnitude of effects varied greatly, ranging from 3.4 to 82.3% for different fungal strains (Chapter 2, 3 and 7). Two treatments, however, resulted in a slight increase in CP, as observed for CS2 (3%; Chapter 2) and LE8-treated MS1 straw (2%; Chapter 7). As discussed in Chapter 4, the relative increase in CP concentration was mainly due to degradation of the cell wall components, as fungi are not able to fix nitrogen (N) from the atmosphere. Based fractionation of CP (Sniffen *et al.*, 1992) and the *in vitro* assessment N availability (IVGP<sub>N</sub>) (Cone *et al.*, 2009), fungi showed a unique ability in metabolizing protein. To my knowledge, the variation between fungi in solubilizing N that benefits ruminants has, hitherto, never been studied. The assessment of these parameters in Chapter 4 was mainly focused on the final changes of the CP fractions and IVGP<sub>N</sub> after 7 weeks of fungal pretreatment. The data of their changes over time, however, were not provided in that chapter, although the measurements were also carried out on samples



**Fig. 8.5.** Time-based changes of the modified *in vitro* gas production (IVGP<sub>N</sub>; A) and selected crude protein fractions (B–D) of wheat straw treated with different fungi for 7 weeks. Dashed-lines indicate control values (at week 0; see Chapter 4) and error bars indicate standard deviations.

for week 1 and 3. Nonetheless, those weekly measurements were included in the statistical models. The changes in IVGP<sub>N</sub> and selected CP fractions over the 7 week of treatment are shown in Fig. 8.5. In general, fungi that showed smaller changes in CP fractions and IVGP<sub>N</sub> at the beginning of the colonization showed lower maximum/minimum values at week 7. Some variations among strains/species of the time-based changes were also noticed. For instance, CS1 showed a decrease in IVGP<sub>N</sub> at week 1, then increased again until week 7; while, CS12 and PE3 showed decreases in IVGP<sub>N</sub> until week 3. The changes in availability of N over time and their relationship to other variables that can be measured, such as enzyme activities/expressions, for a selected fungus can be an interesting subject for future research. This aspect, however, was not covered in Chapter 4 as the focus was more on exploring variations among different fungal strains in the solubilization of protein, using end-point measurements.

### Assessing fungal delignification mechanisms: Accuracy matters

As discussed earlier, the ADL method lead to a serious underestimation of the total lignin content, particularly in fungal-treated wheat straw; hence, limits a complete understanding of the fungal delignification mechanisms. Moreover, no lignin structural information can be obtained from this method. Therefore, Py-GC/MS (both semi-quantitative and quantitative) was carried out on the samples, to quantify the lignin contents (Py.lignin) and simultaneously providing the structural features. Compared to the ADL, the Py.lignin contents were 1.4 to 2.8 times higher in fungal-treated straw (week 7). Interestingly, the Py.lignin content for the untreated straw was even higher (3.7 times), compared to ADL. One may expect higher figures for the fungal-treated straw (instead of control). A likely explanation is the degradation (mineralization) of lignin into CO<sub>2</sub> by fungi. The soluble fraction of lignin in the untreated straw is probably due to sterilization steps (by autoclaving). Autoclaving of the wheat straw prior to the fungal pretreatment is hypothesized to result in a larger fraction of water-soluble lignin and other cell wall polymers.

Through quantitative <sup>13</sup>C-IS Py-GC/MS, most of the extent of delignification by fungi were comparable with the assessment with ADL – except for CS12. The delignification of CS12 was only 27.1%, based on ADL, but Py-GC/MS assessment gave a higher percentage (60.3%). This resulted in an overall species-dependent order of the fungal delignification potential: *C. subvermispora* (~63.3%) > *L. edodes* (~58.0%) > *P. eryngii* (~38.7%), which was in agreement with the observed IVGPs (Chapter 3). In Chapter 5, the lignin subunits and also its structural features were not correlated to total lignin contents and the subsequent IVGP. This clearly indicates a large variation among different fungal strains/species in modifying certain moieties of lignin. To uncover possible unique mechanisms of these fungi, the fungal-treated wheat straws were subjected to a combined analysis of <sup>13</sup>C-IS Py-GC/MS and whole cell wall HSQC nuclear magnetic resonance (NMR) (Chapter 6). It is commonly reported for the preference of fungi to degrade syringyl (S) unit compounds, rather than the guaiacyl (G) units (lower S/G ratio) (Del Río *et al.*, 2002; Van Kuijk *et al.*, 2016). In this thesis, however, there was hardly any clear preference shown by these fungi (Chapter 5 and 6), probably due to extensive degradation of the Py.lignin contents. The largest effect was observed for LE8 (H:G:S = 11:64:25), as compared to control (H:G:S = 10:62:28).

The combined  $^{13}\text{C}$ -IS Py-GC/MS and 2D-NMR analysis gives a novel insight into the fundamental mechanistic differences among fungal species, based on the specific structural moieties. Among the important highlights of this analysis – although *C. subvermispota* and *L. edodes* showed a similar pattern of lignin degradation with time, the remaining lignin structures were greatly differed, confirming the fundamental differences among fungi. It is suggested that the main underlying mechanisms of *C. subvermispota* strains is through the cleavage of  $\text{C}_\beta\text{-O-Aryl}$ , besides  $\text{C}_\alpha\text{-C}_\beta$  cleavage. The former was confirmed by  $^{13}\text{C}$  Py-GC/MS, whereby high increases of  $\text{C}_\alpha$ -oxidized pyrolysis products were observed, particularly vanilloyl acetaldehyde (VAL) and syringoyl acetaldehyde (SAL). It is hypothesized that these acetaldehydes are solely formed from the oxidized  $\beta\text{-O-4}$  linkages. For *L. edodes* and *P. eryngii*, the cleavage of  $\text{C}_\alpha\text{-C}_\beta$  cleavage is the predominant mechanism. Interestingly, the reduction of ferulic acids moieties, which participate in lignin-carbohydrate linkages, was more pronounced in *P. eryngii* treated lignin (30%). No isolation of lignin was carried out in Chapter 6, as normally done to characterize the inter-unit linkages of the ‘pure’ lignin, e.g. as milled-straw lignin (Zeng *et al.*, 2013). The idea of having whole cell wall or *in situ* NMR of the samples was to avoid extensive isolation procedures, which may lead to partial modification of the polymer (Rencoret *et al.*, 2011). Although a severe signal overlapping was observed, particularly for the fungal-treated wheat straw, some substructures such as phenylcoumaran ( $\beta\text{-5}'$ ) and resinol ( $\beta\text{-}\beta$ ) substructures were readily annotated. Other structures such as  $\text{C}_\alpha$ -oxidized ( $\alpha\text{-keto}$ )  $\beta\text{-O-4}'$  and dibenzodioxocins ( $5\text{-5}'/4\text{-O-}\beta$ ) can be observed only at higher zoom levels. For future research, a combined Py-GC/MS and 2D-NMR analysis on the lignin isolates or its various fractions (based on their extractability in water and other solvents) can give a greater detail and accuracy in the delignification mechanism of a particular fungus (on-going research at the Laboratory of Food Chemistry, Wageningen University).

## Concluding remarks

### *Contribution to the current knowledge*

This thesis showed a large variation among different fungal strains in terms of their bioprocessing capabilities, growth characteristics, utilization of a certain nutrient and structural modification of lignin – even if they are grown on the same biomass with similar growth and culture conditions. Despite these variations, the selected fungal strains performed well on different wheat straw types and conditions, as well as across different independent experiments carried out for this thesis. The same experimental setup can be replicated for other biomasses, such as rice straw, oil palm fronds and sugarcane bagasse, either using the identified high performers in this thesis; or using locally available fungal strains/species. Two unique contributions of this thesis to the current knowledge are variability among different fungal strains in solubilizing protein and modifying the lignin structure of wheat straw. The former, in particular, could pave the way to study the inter-relationship between protein and the delignification mechanisms of white-rot fungi. This can also be applied to different fibrous biomasses with an interest on improving its protein availability.

One of the main issues highlighted in this thesis is the difficulty to produce a sound mass balance for all nutrients in the fungal-treated wheat straw, particularly for the cell wall. Since fungi greatly differ in their capabilities to solubilize cell wall components and also growth characteristics, comparisons among different strains/species are difficult. The current methodologies of quantifying lignin and carbohydrate contents cannot provide a satisfactory answer. An accurate assessment of these components is crucial, especially when it comes to bioprocessing of biomass at a larger scale, where the ratio of lignin to carbohydrate losses (i.e. bioprocess efficiency) is an important parameter. However, the interference of fungal biomass in this analysis limits its usability and may be inconclusive. Lignin is the only parameter that can be quantified without (or less) interference of fungal biomass, especially with the accurate  $^{13}\text{C}$ -lignin Py-GC/MS method. Nevertheless, quantifying the lignin content alone may not be sufficient to describe fungal delignification, without providing the changes on its fractions, structural features and the associated cell wall polysaccharides (lignin-carbohydrate complex). The IVGP was exclusively used in this thesis to assess the capability of a particular fungus in the bioprocessing of wheat straw. Although, this method is not specific, due to the marginal contribution of fungal biomass to the total IVGP, it is a highly effective and practical technique to evaluate the end-product of fungal pretreatments.

*Limitation and recommendation for future research*

The experimental setup employed in this thesis was intended to discern the fundamental biological variations among different fungal strains/species; and to select the best performing strains for future research. Due to the limited scope of this thesis, there are many other important elements of the fungal activity that are not covered, such as enzymes, organic acids and secondary metabolites production. These elements are significant for a complete understanding of fungal actions. Considering the large variation observed among different fungal strains, it is worthwhile to perform in-depth studies to uncover the underlying mechanisms of these fungi. Several interventions for future research have been mentioned earlier and will be briefly mentioned here. Two main aspects can be outlined: (1) biological and (2) chemical aspects. The biological aspect is important for an in-depth understanding of fungal action on lignocellulosic biomass. Assessing the enzymatic apparatus of the fungi is one of them and a secretomic or proteomic approach is the only way forward (Salvachúa *et al.*, 2013). The production of organic acids and secondary metabolites are among important biological aspects to understand the underlying cellular mechanisms (Nishimura *et al.*, 2012). Some of the chemical aspects have been covered in this thesis by investigating variations in the modification of lignin structures. To deal with the inaccuracy in mass balance, more advanced techniques have to be considered. The feasibility to quantify fungal biomass grown on the solid substrate needs to be explored, which could be done through labelling of the substrate (or fungi) with stable isotopes ( $^{13}\text{C}$ ) or using a chemical marker on a specific fungal component such as chitin and ergosterol (Wallander *et al.*, 2013). A non-equilibrium thermodynamic approach can also be considered for this purpose (Das and Karim, 1995). Optimization of the Van Soest *et al.* (1991) method for fungal-treated materials should also be considered. This includes improving the extraction and filtration steps involved.

The information from the two aspects can be corroborated to optimize the pretreatment method, e.g. through mathematical modelling, which could expedite the advancement of the bioprocess. This is particularly useful for an industrial application, i.e. solid state fermentation of biomass using fungi that is conducted in a controlled bioreactor. For the on-farm application, the future optimization work (using the selected fungi) should mainly focus on: (1) scaling-up the pretreatment method, which includes sterilization/pasteurization procedures, inoculation and other practical problems; (2) inclusion levels of the fungal-treated biomass in ruminant rations, without causing adverse effects on the animal performance or health.

### Conclusions

The main outcomes of this thesis can be summarized as follows:

- There is a large biological variation among strains belonging to the same fungal species, particularly for their growth characteristics and bioprocessing capabilities. High potential strains of *C. subvermispota*, *P. eryngii* and *L. edodes* were selected, based on the *in vitro* gas production.
- The high potential strains of *C. subvermispota* and *L. edodes* perform well on different wheat straw maturities and cultivars. Nonetheless, the magnitude of ruminal degradability is affected by different cultivars, but not by straw maturities.
- Two strains of *C. subvermispota* differ in their growth and enzyme activity characteristics. Both strains were similar in their capabilities to improve the degradability of wheat straw, although the gas production profiles can be differentiated by curve fitting.
- Fungi are able to solubilize nitrogen from the cell wall and make them more available for fermentation in the rumen. The high potential strains differ in their capabilities to solubilize nitrogen in the substrate. *P. eryngii* and *L. edodes* strains increased the available nitrogen for fermentation, but not *C. subvermispota*.
- Specific analyses of lignin and carbohydrates contents are required for accurate description of the ruminal degradability of wheat straw treated with different fungal strains.
- Each fungal species is fundamentally different in their delignification mechanisms. It is proposed that the cleavage of  $C_{\beta}$ -O-Aryl linkages as the main mechanism for *C. subvermispota*. While the predominant mechanism for *L. edodes* and *P. eryngii* is through the cleavage of  $C_{\alpha}$ - $C_{\beta}$  linkages.

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# Summary

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The use of white-rot fungi in bioprocessing of lignocellulosic biomass such as wheat straw into valuable ruminant feed ingredients is promising. Fungi are capable of degrading lignin – a cell wall polymer that negatively affects ruminal degradability of the biomass. For this purpose, fungi, such as *C. subvermispora*, *P. eryngii* and *L. edodes*, have been identified as potential species. The vast availability of strains of these species, as well as various wheat straw types and conditions lead to four main research questions: (1) Do strains of the same fungal species differ in their extent of lignocellulose degradation and the subsequent improvement in the ruminal degradability of wheat straw? (2) How do high potential strains/species vary in utilizing a certain nutrient, such as protein? (3) Do different fungal strains/species possess unique delignification mechanisms? (4) Do various wheat straw types have effects on how fungi degrade the cell wall components?

As a start to the screening trial, in **Chapter 2**, the bioprocessing capabilities of two strains of *C. subvermispora*, CS1 and CS2, were compared. Both fungi were capable of selectively degrading lignin and improve *in vitro* gas production (IVGP) of wheat straw, with CS1 showing a higher potential than CS2. The two fungi also differed in their growth pattern and enzymatic activities, as well as in the degree of selectivity in lignin degradation. There was no correlation between the activities of the lignin-degrading enzymes and the extent of lignin degradation, indicating that more complex mechanisms are involved in modifying the cell wall. In **Chapter 3**, the IVGP was used as a decisive screening parameter for the best performing fungi. A number of strains of the fungal species *C. subvermispora*, *P. eryngii* and *L. edodes* were screened based of their IVGPs. All strains that belonged to the same species showed a distinctive pattern of growth and changes in the pH of substrate. Nonetheless, large variations of their bioprocessing capabilities were observed. Out of the 32 fungal strains studied, 17 strains showed a significantly higher ( $P < 0.05$ ) IVGP, compared to the control after 7 weeks of treatment. The best performing strains for each species were selected. They were (in order of IVGP for each species): CS1, CS6 and CS12 (*C. subvermispora*); PE6, PE2 and PE3 (*P. eryngii*); LE8, LE7 and LE10 (*L. edodes*).

Two high-ranked strains for each species in Chapter 3 were subjected to three follow-up studies on variation in protein solubilization and modification of lignin structures. In **Chapter 4**, different fractions of crude protein (CP) and *in vitro* protein fermentation of wheat straw treated with six fungal strains (2 strains per species) were evaluated. All fungal strains caused a relative enrichment of the CP content (23.3 to 30.9%) in wheat straw. There was no quantitative difference in the total amount of CP among different fungal-treated straws. However, the CP fraction provided a unique insight into the

variation among fungi in solubilizing protein. The results showed that fungi increased the soluble protein fractions and decreased the cell wall bound protein. Only wheat straw treated with *P. eryngii* and *L. edodes* strains resulted in a higher protein availability for fermentation in the rumen (30.2 to 47.1%). *C. subvermispota* strains on the other hand, did not improve the protein availability. To validate the established relationship between IVGP and the cell wall contents, determined using the Van Soest method (Procedure A), more specific analyses of the cell wall (Procedure B) were used in **Chapter 5**. In Procedure B, pyrolysis (Py-GC/MS) and alditol-acetates were used to determine Py-lignin and carbohydrates content, respectively. Procedure A showed a higher correlation coefficient of lignin/carbohydrate to the IVGP, compared to Procedure B (Pearson's  $r$  of -0.84 vs. -0.69, respectively). The large differences between methods indicate disparities in the mass recovery of cell wall components, which complicates the understanding of degradation of cell wall components and its effect on IVGP. Procedure A is more indicative of the cell wall solubility – which explained the IVGP well, rather than indicating the actual cell wall degradation by fungi. The more specific Procedure B better elucidates the structure, which helps with the understanding of variations among fungal strains in modifying the cell wall. As for the cellulose (and glucan) contents, neither procedure resulted in an accurate quantification of the cellulose due to interference of fungal biomass. A contribution of pure fungal biomass to the total IVGP was expected, since it showed higher IVGP than its corresponding treated wheat straw. Hence, specific analyses of the lignin and carbohydrates contents are required for accurate description of the ruminal degradability of wheat straw.

The combined pyrolysis-GC/MS and 2D-nuclear magnetic resonance (NMR) analysis in **Chapter 6**, revealed fundamentally different delignification mechanisms among fungal species. It was proposed that the cleavage of  $C_{\beta}$ -*O*-aryl linkages as the main mechanism for *C. subvermispota*, besides  $C_{\alpha}$ - $C_{\beta}$  cleavage. While the predominant mechanism for *L. edodes* and *P. eryngii* was through the cleavage of  $C_{\alpha}$ - $C_{\beta}$  linkages. In **Chapter 7**, the persistence of the high potential strains of *C. subvermispota* and *L. edodes* in improving the ruminal degradability of wheat straw of different maturities and cultivars was studied. The degradation of lignin by both fungi was more pronounced on mature straw (MS3; ~89.0%), compared to the MS1 (~70.7%). In contrast, cellulose was less degraded on MS3 compared to MS1 (~8.3 vs. 14.6%). The 'preference' of fungi in the delignification of a more mature straw was confirmed by Py-GC/MS. A higher abundance of the S-unit compounds was found on MS3, which made the mature straw more susceptible to fungal attack. A variable extent of degradation of lignin (47 to 93.5%) was

observed when both fungi were grown on different straw cultivars. The high potential strains of *C. subvermispota* and *L. edodes* were able to improve the IVGP of various straw types. However, the magnitude of IVGP was only affected by different cultivars, but not by straw maturity.

Research presented in this thesis discerned the biological variation of different fungal strains/species in modifying the cell wall contents, producing variable effects on the ruminal degradability of wheat straw. The new finding on the capability of some fungi to solubilize protein from the cell wall is important, which could pave the way for more advanced and in-depth studies in the future. The high potential strains selected in Chapter 3 were proven capable of improving the nutritive value of various batches of wheat straw, indicating the potential of bringing this eco-friendly pretreatment method into practice.

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Sincerely,

*Nazri*

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# About the author

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Résumé • Publication • Training & supervision plan

## Résumé: About the author

**M**ohd Nazri Bin Md Nayan was born on 7<sup>th</sup> May 1986 in Pahang, Malaysia. Born to oil palm farmer parents, he received his early education at FELDA Sungai Retang Primary School, Jerantut, Pahang, before continuing his secondary education at Sultan Haji Ahmad Shah Science School, Pekan, Pahang. In 2008, he obtained a BSc in Biomedical Sciences from Universiti Putra Malaysia (UPM), Serdang, before pursuing an MSc degree at the same university in Animal Physiology. During his MSc study, he was awarded Erasmus-Mundus scholarship to do a research internship at Universitat Autònoma de Barcelona, Spain for 10 months. His research was about supplementing soybean oil to alleviate effect of heat stress on dairy goats by assessing the urine metabolomics. After completing his MSc study, he started his PhD study at the Animal Nutrition group, Wageningen University & Research (WUR) from September 2014. He was involved with “More Meat and Milk from Straw” project, which focused on variation among different fungal strains in bioprocessing of wheat straw into ruminant feed. The results of his PhD study are presented in this thesis. Currently, he is working as a researcher cum laboratory technician at the Animal Nutrition group, WUR. When returning to his home country, he will resume his job as an assistant lecturer at the Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia.



## Publications

- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2017). Differences between two strains of *Ceriporiopsis subvermispora* on improving the nutritive value of wheat straw for ruminants. *J. Appl. Microbiol.* **123**, 352–361.
- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2018). Screening of white-rot fungi for bioprocessing of wheat straw into ruminant feed. *J. Appl. Microbiol.* **125**, 368–379.
- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2018). Variation in the solubilization of crude protein of wheat straw by different white-rot fungi. *Anim. Feed Sci. Technol.* **242**, 135–143.
- Nayan, N., Van Erven, G., Kabel, M.A., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2018). Improving ruminal digestibility of various wheat straw types by white-rot fungi. *J. Sci. food Agric.* In press.
- Van Erven, G., Nayan, N., Kabel, M.A., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2018). Mechanistic insight in the selective delignification of wheat straw by three white-rot fungal species through quantitative <sup>13</sup>C-IS py-GCMS and whole cell wall HSQC-NMR. *Biotechnol. Biofuels*, **11**, 1-16.
- Nayan, N., Van Erven, G., Kabel, M.A., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. The relevance of different analytical methods to assess fungal degradation of lignocellulose and explaining the subsequent ruminal degradability. Submitted to *J. Sci. Food Agric.*

## Conferences and Symposia Proceedings

- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2016). Evaluation of two strains of *Ceriporiopsis subvermispora* on improving the nutritive values of wheat straw for ruminant feed. WIAS Science Day 2015, 4 February, Wageningen, the Netherlands.
- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2016). Evaluation of *Ceriporiopsis subvermispora* on improving the nutritive value of wheat straw. Proceedings 67<sup>th</sup> Annual Meeting of the European Federation of Animal Science, 29 August, Belfast, United Kingdom.

- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2017). Selection of white-rot fungi for bioprocessing of wheat straw into ruminant feed: Preliminary results. Proceedings 42<sup>nd</sup> Animal Nutrition Research Forum, 7 April, Gent, Belgium.
- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2017). Selection of White-Rot Fungi for Bioprocessing of Wheat Straw into Ruminant Feed. Proceedings First ASAS-CSAS Annual Meeting & Trade Show, 8 July, Baltimore, MD, USA.
- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2018). Variation in the solubilization of nitrogenous compounds in wheat straw by different white-rot fungi. Proceedings 43<sup>rd</sup> Animal Nutrition Research Forum, 11 April, Wageningen, the Netherlands.
- Nayan, N., Sonnenberg, A.S.M., Hendriks, W.H. and Cone, J.W. (2018). Variation in the solubilization of nitrogenous compounds in wheat straw by different white-rot fungi. Proceedings 69<sup>th</sup> Annual Meeting of the European Federation of Animal Science, 28 August, Dubrovnik, Croatia.

## The Basic Package (3 ECTS)<sup>2</sup>

WIAS Introduction Day	2014
WIAS Ethics and Philosophy of Life Science	2014
WIAS Course on essential skills	2014

## Disciplinary Competences (10 ECTS)

Writing research proposal	2015
WIAS Design of Experiment	2015
WIAS Statistics for Life Sciences	2016
Advances in Feed Evaluation Science	2017

## Professional Competences (11 ECTS)

High-Impact Writing in Science	2015
Techniques for Writing and Presenting a Scientific Paper	2015
Project and Time Management	2016
Interpersonal Communication for PhD Students	2016
Scientific Publishing	2017
Brain Training	2017
WAPS council	2017
Effective behavior in your professional surroundings	2016
The Final Touch: Writing the General Introduction and Discussion	2018

<sup>1</sup> Completed in the fulfilment of requirements for the education certificate of the Graduate School, Wageningen Institute of Animal Science (WIAS)

<sup>2</sup> One ECTS equals a study load of 28 hours

**Presentation Skills (4 ECTS)**

WIAS Science Day, Wageningen, Netherlands; poster	2016
European Federation of Animal Science, Belfast, UK; poster	2016
Animal Nutrition Forum, Belgium; oral	2017
ASAS-CSAS Annual Meeting, USA; oral	2017
Animal Nutrition Forum, Wageningen; oral	2018
European Federation of Animal Science, Dubrovnik, Croatia; oral	2018

**Teaching competences (6 ECTS)**

Supervising MSc thesis	2016
Supervising BSc Thesis	2017
Supervising practical - BSc course "Principles of Animal Nutrition"	2016-2018

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**Total ECTS****34**

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## Colophon

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