Storage of fungal treated lignocellulosic biomass and its acceptance by goats

Lei Mao

Propositions

1. *Ceriporiopsis subvermispora* and *Lentinula edodes* treatment makes lignocellulosic biomass into a safe, more nutritious and storable ruminant feed.
   (this thesis)

2. As with many good wines, the palatability of *Ceriporiopsis subvermispora* and *Lentinula edodes* treated wheat straw for caprine increases with storage.
   (this thesis)

3. Writing a good scientific paper is like telling a good story.

4. Statistically significant results are not required for a good research paper.

5. The working efficiency of a person depends on the enthusiasm for the job.

6. Regular and moderate physical labour improves the creativity of the mind.

Propositions belonging to the thesis, entitled:

'Storage of fungal treated lignocellulosic biomass and its acceptance by goats'

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Chapter 1

General introduction

1.1 Background

As the world population and living standard continue to increase, the demand for animal-based food products and energy resources is also rising. These trends intensify the competition of land use for the production of human food, biofuels and animal feed and feed ingredients. To meet these changes, it is essential to increase our attention to the increased use of agricultural organic residues, which can make a significant contribution to future demands in animal-based food (Van Kuijk *et al*., 2015a; Rouches *et al*., 2016). Agricultural activities result in an enormous accumulation of plant-derived by-products (e.g. various cereal straws, sugarcane bagasse, oil palm fronds, etc.), which is only partly reused. Among them is wheat straw which is widely available throughout the world and abundant in cell wall carbohydrates. Regardless of the origin, sterilized wheat straw comprises of 46-50% cellulose, 26-32% hemicellulose and 6-9% lignin (Tuyen *et al*., 2012; Van Kuijk *et al*., 2015b; Nayan *et al*., 2017, 2019). Lignin forms a rigid barrier, and prevents the cell wall carbohydrates from being metabolized by rumen microbes. The amount of acid detergent lignin (ADL) in plant cell walls is, therefore, negatively correlated to microbial fermentation (Jung and Vogel, 1986; Arora and Sharma, 2009). As a consequence of the low nutritional value for animals and little commercial value, most of these plant-derived by-products are ploughed back into the soil or burned. The latter results in addition to inefficient use of natural resources, to environmental pollution. Compared with non-biological pre-treatment, pre-treating with white-rot fungi is considered an efficient and environmentally friendly method to break down lignin in such a way that the carbohydrates (cellulose and hemicellulose) in biomass become available to hydrolytic enzymes (Rouches *et al*., 2016; Van Kuijk *et al*., 2015a). Several studies have been conducted on a laboratory scale, showing that especially with the use of selective white-rot fungi, a large reduction in lignin and hemicellulose is obtained with little loss of cellulose leading to an increase in fermentability by rumen microbes (Tuyen *et al*., 2012, 2013; Van Kuijk *et al*., 2015b). However, there are still a number of issues to be addressed for large scale application of this bio-technology, such as the safety of the fungal treated wheat straw to ruminants, stability during long term storage and the acceptance by ruminants. All the latter aspects are the subjects of this thesis.

1.2 Plant cell wall structure

Plant cell walls are essential for the survival and development of the plant, and are

important for the physiological activity of the cell, integrity of the structure and strength and flexibility of the plant. Plant cell walls consist of three major layers with a complex macromolecular structure, the primary cell wall, the secondary cell wall and the middle lamella. During growth the cell wall consists only of the primary wall. During maturation the secondary wall is synthesized inside of the primary cell wall (Kirk and Cullen, 1998). Lamella, which are rich in pectins, located between two cells are essential for connection between the adjacent cells.

The secondary cell wall is mainly composed of cellulose, hemicellulose and lignin, which typically make up more than two thirds of the cell wall components. The cell wall also contains small amounts of protein, pectins, starch and soluble sugars (e.g. fructose and glucose) (Bidlack, *et al*., 1992; Grabber, 2005; Shan *et al*., 2008; Tishler *et al*., 2015). Cellulose is a linear polysaccharide of D-glucose sub-units linked by β-1,4 glycosidic bonds. These linear chains are grouped together and form microfibrils, which is the unit of cellulose fibrils (Yu *et al*., 2008). Hemicellulose is a heterogeneous polysaccharide composed of pentose (xylose and arabinose), hexose (mannose, glucose and galactose) and may contain sugar acids, the content of which vary between different biomasses (Pérez *et al*., 2002; Saha, 2003). Hemicellulose in straw mainly consist of xylan, and lesser amounts of arabinan, mannan and galactan (Salvachúa *et al*., 2011). In comparison with cellulose, hemicellulose is more readily hydrolysed due to its amorphous structure. Lignin is an aromatic heteropolymer, composed of three main phenylpropane units, being hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (Bugg *et al*., 2011). Wheat straw contains ca. 24% lignin of which 22.8% is acid-insoluble and 1.2% acid-soluble (Salvachúa *et al*., 2011). The amount and composition of lignin differ between types of biomass and depends on the stage of maturation. The linkages (ether and C-C) present in lignin makes lignin highly resistant to hydrolytic attacks. Lignin does not exist as an independent polymer, it is chemically bound to hemicellulose, which is cross-linked with cellulose. The structural complexity restricts the accessibility of hydrolytic enzymes to the structural carbohydrates.

1.3 Common methods to degrade lignin

Utilization of lignocellulosic biomass as animal feed or industrial production requires an efficient pre-treatment to disrupt the lignocellulose structure and to liberate the polysaccharide fractions. Pre-treatment methods can roughly be classified into physical,

chemical and biological methods. Pre-treatment methods are usually applied in combination, for example thermal and chemical, thermal and biological or multiple treatments, and are defined as physicochemical or biochemical methods as described in many reviews (Mosier *et al*., 2005; Prasad *et al*., 2007; Agbor *et al*., 2011; Balat, 2011). Physical processes, like mechanical chopping, milling, pelleting and grinding are usually used to reduce the particle size, cellulose crystallinity and polymerization of the substrate to increase the surface area for a better enzymatic hydrolysis. Other methods like hot water and steam explosion pre-treatment can also open up the fibres in the cell wall and make these more accessible for subsequent processes, such as fermentation and enzymatic hydrolysis. These methods require high pressure at high temperatures (above 150 °C) and sometimes need the addition of catalysts (e.g. H_2SO_4) to enhance the efficiency of the process. Chemical methods, such as acid (e.g. sulfuric acid and hydrochloric acid) and alkali (e.g. sodium hydroxide) treatment are used to hydrolyse hemicellulose and remove lignin and hence increase the accessibility of cellulose. All of these methods are effective in increasing the accessibility of carbohydrates (mainly cellulose). However, these methods are often more applicable at an industrial scale than on a single farm, because of a high demand for facilities and energy. The demand also highly depends on the biomass characteristics (e.g. lignin content) and the final product required. For acid or alkali pre-treatment, neutralization of the pH is needed for further use in feeds or enzymatic hydrolysis. In addition, some chemicals are toxic and corrosive, hence are potential hazardous to animals, farmers and the environment. As a consequence of various pre-treatments, some by-products released from lignocellulose (e.g. phenolic components) degradation might also have an inhibitory effect on fermentation or rumen microbes (Jönsson and Martín, 2016). The harsh thermal pre-treatment may result in the re-condensation and precipitation of soluble lignin compounds and increase the cellulose crystallinity (Hendriks and Zeeman, 2009), hence influence further microbe degradation of lignocellulose. Biological methods comprise mainly of pre-treatment by white-rot fungi. White-rot fungi are capable of decaying cell walls through enzyme or non-enzyme systems and can be very selective and efficient. However, pre-treating with white-rot fungi also has disadvantages, such as a long incubation period required, infections of the substrate with unwanted microorganisms, requirement for equipment and dry matter lost during the fungal treatment. An ideal pre-treatment method should be efficient, low cost, environmentally friendly, applicable in a variety of environments and on any scale and

should not produce compounds that have an inhibitory effect on downstream processes or use. None of the methods mentioned above currently meet all the requirements or meet them to the same extent. Also pre-treating with white-rot fungi has its drawbacks, but as an environmentally friendly method with low cost it has certainly potential as a pre-treatment method to bio-convert lignocellulosic biomass to a higher valued animal feed ingredient.

1.4 Using white-rot fungi as degrader

The majority of the wood decay activity on earth is conducted by aerobic wood-degrading fungi, which are classified based on their degradation pattern as white-, brown- and soft-rot fungi (Hatakka and Hammel, 2011; Wan and Li, 2012). During the vegetative growth, the mycelium colonizes the substrate to open the plant cell walls. The metabolic activity of the fungi results in the depolymerisation of lignin, making cellulose and hemicellulose available to be used by the fungus later. White-rot fungi have either a non-selective or selective pattern of degradation. White-rot fungi classified as non-selective fungi are able to decompose lignin and utilize simultaneously cellulose and hemicellulose. The selective fungi degrade lignin and hemicellulose during their vegetative stage but use little or no cellulose. Therefore, the selective white-rot fungi show the greatest potential to be used as a pre-treatment of lignocellulosic biomass. However, even with selective white-rot fungi, the mode of action varies greatly between fungal species, strains and substrates (Tuyen *et al*., 2012, 2013; Van Kuijk *et al*., 2015b; Nayan *et al*., 2018, 2019). Tuyen *et al*. (2012) studied a number of white-rot fungi when grown on wheat straw, showing that *C. subvermispora* and *L. edodes* are two highly promising fungi improving the rumen fermentability of the substrate with a low dry matter and cellulose loss after seven weeks of incubation in comparison to other fungal species. Nayan *et al*. (2018) investigated 12 strains of *C. subvermispora* and 10 strains of *L. edodes* and *P. eryngii*, each growing on wheat straw, showing that within a species large differences in growth and lignin degradation occur.

Manganese peroxidases (MnPs), lignin peroxidases, versatile peroxidases and laccases are commonly reported oxidative enzymes involved in lignin degradation (Pollegioni *et al*., 2015). Manganese peroxidases catalyse Mn^{2+} to Mn^{3+}, which oxidizes the phenolic compounds in combination with carboxylic acids. The phenolic part only accounts for a minor amount of lignin in the biomass. Recent research indicates that MnPs, with the

involvement of carboxylic acid, are also involved in the degradation of non-phenolic lignin (Qin *et al*., 2017). Lignin peroxidases are effective on non-phenolic lignin, which accounts for up to 90% of the lignin (Pollegioni *et al*., 2015). Laccases also oxidise phenolic compounds and simultaneously convert O_2 into H_2O (Shleev *et al*., 2006; Pollegioni *et al*., 2015). The dominant enzymes secreted by *C. subvermispora* and *L. edodes* are MnPs and laccases (Hatvani and Mécs, 2002; De Souza-Cruz *et al*., 2004; Fernández-Fueyo *et al*., 2012; Nayan *et al*., 2017). The genes involved in lignin degradation for these two fungi have been described previously (Rajakumar *et al*., 1996; Fernández-Fueyo *et al*., 2012; Chen *et al*., 2016).

Although activity of enzymes is a prerequisite for an efficient degradation process, these enzymes are not able to penetrate the intact or early decayed cell walls to reach lignin (Srebotnik *et al*., 1988; Daniel *et al*., 1990; Blanchette *et al*., 1997). The reason is that the molecular mass of the lignin degrading enzymes like MnPs (~ 40-50 kDa) (Pollegioni *et al*., 2015) is too large to penetrate through the micro pores of intact cell walls (Blanchette *et al*., 1997). The hyphae are mainly located on the surface of the lumen in the very beginning of degradation. The erosion starts from the cell lumen, and degrades the cell walls from the secondary cell wall to the middle lamellae (Blanchette *et al*., 1997). Although the permeability of early decayed wood changes after a fungal treatment (Blanchette *et al*., 1997), the slight increase in pore size after the fungal treatment does not enable enzymes to diffuse deeper into the cell wall. Enzyme diffusion only occurs when the cell walls are structurally modified to a large extent or degraded (Srebotnik *et al*., 1988; Blanchette *et al*., 1997). The direct contact between a fungal hyphae and a cell wall is not necessary for the degradation process. To reach and degrade the lignocellulosic matrix from a distance, diffusible low molecular radicals are produced that diffuse into the dense lignin structure and effectively depolymerise phenolic and non-phenolic lignin (Hammel *et al*., 2002; Ohashi *et al*., 2011; Qin *et al*., 2017).

1.5 The safety of fungal treated wheat straw as animal feedstuff

Safe feed for animals and food for humans is of paramount importance. Mycotoxins are a group of secondary metabolites produced by filamentous fungi (Pandey *et al*., 2000; Streit *et al*., 2012). These mycotoxins can be produced during growth in the field and during storage (Streit *et al*., 2012). Even low concentrations of mycotoxins in animal feed may cause serious toxic responses in the animals, and thus threaten the human health

through consuming meat and milk (Streit *et al*., 2012). The fungus *L. edodes* is an edible mushroom (Shiitake) and the compounds in this mushroom have thoroughly been explored (Chen *et al*., 2015), and it is a fungus that is generally regarded as safe (GRAS status). *C. subvermispora* does not produce fruit bodies. To my knowledge, no study has reported the presences (or absence) of common mycotoxins which may be produced by *C. subvermispora*.

1.6 Storage of fungal treated wheat straw by mimicking ensiling

To achieve the maximum *in vitro* degradation of the wheat straw in rumen fluid by *C. subvermispora* and *L. edodes*, several weeks of incubation are needed (Tuyen *et al*., 2012; Van Kuijk *et al*., 2015b). During the initial growth phase these fungi use the free sugars and easy degradable compounds, while during the subsequent incubation weeks, these fungi change to degradation of hemicellulose to sustain the formation of fungal biomass. In general, the longer the incubation period with fungi, the more lignin is degraded, but with a simultaneous loss of the carbohydrates (cellulose and hemicellulose), which mainly contribute to the *in vitro* degradation (Van Kuijk *et al*., 2015b). Therefore, terminating the fungal activity at the right time to optimise the nutritive value for further use as a feed ingredient is essential. In addition, as often large quantities of biomass are available during a short period of time (e.g. at harvest of wheat or rice), the fungal treated biomass once suitable for use as a feed ingredient, has to be preserved as the feeding of animals is a more continues process where smaller amounts are provided to the animals on a daily basis.

Ensiling is a method that is widely used for the preservation of forages for ruminant animals. The process arises as a result of epiphytic lactic acid bacteria which anaerobically ferment water soluble carbohydrates to produce organic acids (mainly lactic acid), which cause a decrease in pH, hence preserving the forage when stored anaerobically. Since lactic acid bacteria play the dominant role in the fermentation process, the process is strongly affected by the presences and the species of lactic acid bacteria, available soluble sugars, absence of oxygen and temperature. Therefore, in the case of forages containing insufficient lactic acid bacteria and sugars, addition of lactic acid bacteria and sugars may be required. During the initial phase of ensiling, the existing oxygen is consumed by plant respiration and aerobic microorganism activity. Therefore,

the ensiling process is more than only the fermentation by lactic acid bacteria, but a fermentation process with different microorganisms.

In the present work, I tried to store *C. subvermispora* and *L. edodes* treated wheat straw, via the principle of ensiling. Although the two white-rot fungi are capable of secreting acids and decreasing the pH, as reported in many studies (Galkin *et al*., 1998; Aguiar *et al*., 2006; Kwak *et al*., 2016), it is not known if those acids are sufficient for the storage of the biomass over extended periods of time. Studies on the combination of fungal bioconversion and the ensiling process are rare. Yang *et al*. (2001) reported of an experiment with corn straw (with 10% wheat bran) treated with *Penicillium decumbens* and mixed with lactic acid bacteria and molasses, and stored in glass jars at a laboratory scale. In the latter experiment the pH decreased from 6.3 to 4.5 during the first day of the ensiling process. This means that the major decrease in pH is not the result of the organic acids produced by the fungi during the aerobic incubation, but mainly from the activity of the lactic acid bacteria under the anaerobic conditions. The possibility to conserve the fungal treated wheat straw without additives was not reported by Yang *et al*. (2001). Thomsen *et al*. (2016) reported a combination of a fungal treatment and ensiling, who first ensiled wheat straw with lactic acid bacteria and then treated the biomass with fungi. The storage of fungal treated biomass might persist at different temperatures for several weeks or month depending on local environmental temperatures. During the ensiling process, the storage temperature is a key factor that can influence the silage quality by affecting the activity of the microorganisms, mainly the lactic acid bacteria. It is important, therefore, to determine the effect of storage temperature on the quality of fungal treated biomass.

1.7 Fungal treated wheat straw as animal feed in practice

A feed needs to be first consumed to be of any nutritional value. As such, the acceptance of fungal treated substrates by animals is essential, since it directly determines the intake rate of the animals. Feeding white-rot fungi treated agricultural biomass to ruminants has been investigated (Shrivastava *et al*., 2012; Oguri *et al*., 2013), but the preference of these novel feeds by ruminant animals has, to my knowledge, never been tested before. A variety of factors affect the preference or aversion of a substrate by an animal, such as nutritional content, flavour, smell, taste, post-ingestive consequences, past experience and toxins (Morand-Fehr, 2003; Provenza *et al*., 2003). As described above, white-rot fungi

greatly increase lignin decomposition and *in vitro* digestibility of the treated substrates (Tuyen *et al*., 2012; Van Kuijk *et al*., 2015b), but they may also produce a variety of secondary metabolites (e.g. organic acids), which may affect the acceptance by animals. To my knowledge, there is no study to date in the literature focussing on the preference of fungal treated wheat straw, compared with "usual" feed, such as grass silage and maize silage by ruminant animals.

1.8 The importance of a faster colonization

A fast and complete colonization of fungi on lignocellulosic biomass is a prerequisite for a successful fungal treatment, because it can, to some extent, protect the biomass from being contaminated during early incubation. At a laboratory scale, autoclaving (121 °C for 1 h) is the most common method used to ensure an uncontaminated growth environment for fungi (Tuyen *et al*., 2012, 2013; Van Kuijk *et al*., 2015b). With this thermal pre-treatment, *C. subvermispora* and *L. edodes* require 2-4 weeks to fully colonize the wheat straw. However, autoclaving is less applicable in practice for small scale farmers, due to the expensive facilities and energy requirements. A more applicable alternative with lower temperatures and a faster colonization is, therefore, essential for this bio-technology to be applied in practice. The carbon absorbed by fungi is mainly used for respiration and mycelial growth (Manzoni *et al*., 2012). The higher production of fungal biomass, therefore, depends on a higher carbon use efficiency (CUE), which is defined as the ratio of carbon used for growth of new fungal biomass and carbon consumed (Manzoni *et al*., 2012). Wheat straw contains a large amount of structural carbohydrate but limited soluble nutrients. However, a low CUE is expected because fungi must degrade lignin to liberate and utilize cell wall carbohydrates. Wheat bran, a by-product of wheat milling containing hemicellulose, starch and protein, and less cellulose and lignin, has the potential to serve as a carbon source for fungi. In addition, wheat bran contains more nitrogen, often a factor for growth of fungi (Tudzynski, 2014). Addition of wheat bran to wheat straw might have a positive effect on fungal colonization and lignin degradation.

1.9 Thesis aim

This thesis combines research that contributes to the implementation of fungal treatment of biomass in practice. In all experiments, wheat straw (organic and conventional) was

used as the biomass of interest. One of the main aims of the research described in this thesis was to explore the possibility to store fungal treated wheat straw under anaerobic conditions. In **Chapter 2**, two separate experiments are described. In the first experiment, conventional wheat straw was incubated with *C. subvermispora* and *L. edodes*, and changes in pH, chemical composition and *in vitro* degradability of the wheat straw were investigated. In the second experiment, wheat straw, autoclaved wheat straw, *C. subvermispora* and *L. edodes* treated wheat straw were stored without additives, with the addition of lactic acid bacteria, and with the addition of a combination of lactic acid bacteria and molasses. Also the quality of the treated wheat straw was evaluated. **Chapter 3** aimed to determine the compounds generated as incubation progresses. Mycotoxin analyses were performed to assess this aspect of the safety of fungal treated wheat straw for animal consumption. **Chapter 4** aimed to determine the effect of different temperatures on the quality of stored fungal treated wheat straw. Organic wheat straw was incubated with *C. subvermispora* and *L. edodes* for 7 weeks and then stored at temperatures up to 52.4 °C. Changes in pH, titratable acidity, chemical composition and *in vitro* degradability were determined. Since the colour of the stored fungal treated wheat straw was different with the increase in temperature, colour differences were quantified. The final aim of a successful fungal treatment and its preservation is the utilization as a feed or feed ingredient. **Chapter 5** aimed to determine the preference of fresh and stored fungal treated wheat straw relative to more commonly used feed ingredients in the Netherlands (grass and maize silage) when fed to goats. Since the efficiency of lignin degradation by fungi is affected by the speed of colonization, an experiment was performed by adding different amounts of wheat bran to the biomass (**Chapter 6**).

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Chapter 2

Preservation of *Ceriporiopsis subvermispora* and *Lentinula edodes* treated wheat straw under anaerobic conditions

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Abstract

No attention has been paid so far to the preservation of fungal treated lignocellulose for longer periods. In the present study, we treated wheat straw (WS) with the white-rot fungi *Ceriporiopsis subvermispora* and *Lentinula edodes* for 8 weeks and assessed changes in pH, chemical composition and *in vitro* gas production (IVGP) weekly. Fungal treated WS was also stored for 64 days 'as is', with the addition of lactic acid bacteria (LAB) or with a combination of LAB and molasses in airtight glass jars mimicking ensiling conditions. Both fungi significantly reduced the lignin and hemicellulose content of WS, and increased the cellulose content. The IVGP increased with increasing time of incubation, indicating the increase in digestibility. Both fungi lowered the pH of WS under 4.3, which guarantees an initial and stable low pH during anaerobic storage. Minor changes in fibre composition and IVGP were observed for stored *L. edodes* treated WS, whereas no change occurred for *C. subvermispora*. It is possible to conserve *C. subvermispora* and *L. edodes* treated straw under anaerobic condition without additives up to 64 days. This finding is important for practical application to supply fungi treated feed to ruminant animals for a prolonged period.

Key words

Ceriporiopsis subvermispora; *Lentinula edodes*; wheat straw; *in vitro* gas production; anaerobic storage.

2.1 Introduction

Large quantities of agricultural by-products (e.g. rice and wheat straws) are produced every year and most of it is left on the field or burned. However, both rice and wheat straws (WS) can be potential valuable feedstuffs for ruminants, as the major part consists of cellulose and hemicellulose (Prasad *et al*., 2007; Sarkar *et al*., 2012). These carbohydrates are to a limited extent used by rumen microorganisms because cereal straw contains high levels of lignin with complex linkages to the carbohydrates (Buranov and Mazza, 2008). Lignin itself cannot be degraded under anaerobic conditions in the rumen and its concentration is negatively correlated with cell wall degradability (Jung and Vogel, 1986). Therefore, different methods have been used to make the carbohydrates more accessible to rumen microbiota, including physical, chemical, physicochemical and biological methods (Sarnklong *et al*., 2010; Sarkar *et al*., 2012). A promising low tech and low cost method for upgrading low value, high lignocellulose-containing biomass is the selective lignin degradation by white-rot fungi (Tuyen *et al*., 2012, 2013; Van Kuijk *et al*., 2015a). White-rot fungi are the only organisms that are able to degrade lignin effectively by producing extracellular enzymes, such as lignin peroxidase, manganese peroxidase and laccase (Higuchi, 2004). Studies found that the nutritive value and degradability of cereal straw improved after a fungal treatment (Tuyen *et al*., 2012, 2013; Van Kuijk *et al*., 2015b). Fungi can degrade lignin aerobically during an incubation period of several weeks depending on the species and fungal strain. After successful lignin degradation, termination of fungal activity is essential to maintain the nutritive value for ruminants. In addition, to make fungal treated biomass available as a feed ingredient for ruminants over a prolonged period of time, successful conservation is essential.

Ensiling is widely used as a method to preserve forages, such as grass and maize for ruminants. The process of ensiling is based on the anaerobic fermentation of sugars by lactic acid bacteria (LAB), which produce lactic acid and decrease the pH to around 4. The acidic environment effectively inhibits the proliferation and fermentation of other undesirable microorganisms, such as yeasts, enterobacteria and clostridia. Forages can be well preserved with minimum nutritive losses during ensiling (Muck, 1988) and the palatability increases by the formation of lactic acid. The number of lactic acid bacteria and amount of soluble sugars are important to achieve a fast decrease in pH in the silage. However, fermentation might be hampered for substrates with a low content of epiphytic

LAB and sugar. In those cases, LAB and molasses (sugars) can be added to the silages (Umana *et al*., 1991; Lima *et al*., 2010).

The present study aimed to determine the possibility to store fungal treated WS under anaerobic conditions 'as is', with the addition of LAB or with a combination of LAB and molasses.

2.2 Materials and methods

2.2.1 Fungal strains and spawn preparation

The fungi *Ceriporiopsis subvermispora* (strain code: CBS 347.63; Origin: USA) and *Lentinula edodes* (strain code: CCBAS389; Origin: Czech Republic) were selected for the present study because they have been shown to have a greater ability to degrade lignin in lignified biomass (including WS) compared to other investigated white-rot fungi (Tuyen *et al*., 2012). The spawn was prepared as described previously by Van Kuijk *et al*. (2015b).

2.2.2 Fungal solid state fermentation and storage study

Conventional WS was used as substrate for the solid state fermentation with the two fungi. The straw was chopped to a length of approximate 0.5 cm and submerged in water for 3 days, after which the water was drained over a 5 h period. The wet WS was mixed, distributed into plastic containers (3000 ml, with cover: 195 × 195 mm, base: 185 × 185 mm, height: 112 mm; model TP3000 + TPD3000; Combiness, Nazareth, Belgium) containing a filter and autoclaved at 121 °C for 1 h. After cooling to room temperature, 12-13 g (10% of dry WS) of spawn was added to each box and mixed gently by hand under aseptic conditions. In the first experiment, each container was filled with approximately 544 g of wet WS [dry matter (DM) content of autoclaved WS (AWS) was 190.7 g kg^{-1}]. The containers were incubated in a climate controlled room (24 °C) for 8 weeks, and three containers inoculated with each fungus were collected each week to determine pH, chemical composition and *in vitro* gas production (IVGP).

In the second experiment, each container was filled with approximately 541 g (*C. subvermispora*) and 531 g (*L. edodes*) wet WS of the same batch as Experiment 1. The DM content of the AWS was 180.2 g kg^{-1}. Wheat straw was incubated with *C. subvermispora* and *L. edodes* for 39 and 52 days, respectively. Untreated WS, AWS (121 °C for 1 h) and AWS treated with *C. subvermispora* and *L. edodes* were then packed

into 500 ml airtight glass jars 'as is', with 1×10^6 colony-forming units *Lactobacillus plantarum* g^{-1} wet substrate or with a combination of LAB and molasses (3% wet weight). The jars were filled by pressing the substrate into the jars, leaving as little air as possible before being closed and stored at 20 °C in a climate controlled chamber. Three jars of each treatment were collected at 0, 2, 4, 8, 16, 32 and 64 days of storage for analysis with pH being determined directly in all collected samples as described below. Chemical analysis was conducted on air dried samples while volatile fatty acids (VFA) and ammonia (NH_3-N) were determined on fresh samples from day 0 and 64.

2.2.3 Analytical methods

Samples were air dried at 70 °C until constant weight and ground in a hammer mill over a 1 mm sieve (Peppink 100 AN; Peppink Mills BV, Olst, The Netherlands). The DM content was determined after drying at 103 °C for 4 h (ISO 6496, 1999) and ash by incineration at 550 °C for 3 h (ISO 5984, 2002). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined by the methods of Van Soest *et al*. (1991), using an Ankom fiber analyzer (ANKOM 2000 I fibre analyzer; ANKOM Technology, Macedon, NY, USA). In short, NDF was determined by boiling the dried material with ND-reagent with the addition of a heat stable amylase. The insoluble residue was designated as the NDF fraction. ADF comprised the insoluble fraction after boiling the material in AD-reagent. The ADF fraction was subsequently incubated with 72% sulphuric acid for 3 h at 20 °C and the insoluble fraction was designated as ADL. Each fibre fraction was corrected for ash content. Cellulose was calculated as the difference between ADF and ADL and hemicellulose as the difference between NDF and ADF. Nitrogen content was determined using the Kjeldahl method with $CuSO_4$ as catalyst and crude protein (CP) was calculated as N × 6.25 (ISO 5983, 2005).

Next, 30 g of fresh straw was weighed into a stomacher bag and 270 ml demineralised (demi) water was added, followed by mixing in a stomacher (400 Circulator; Seward, Worthing, UK) at 230 rpm for 5 min after which the pH was measured (Model HI 9024; Hanna Instruments, IJsselstein, The Netherlands). In the second experiment, the samples were treated in the same manner, 0.6 ml of liquid was collected and mixed vigorous with an equal volume (1:1, v/v) of trichloroacetic acid (10%) for analysis of NH_3-N, or for analysis of VFA mixed with an internal standard solution (85% ortho-phosphoric acid

containing 19.681 mmol l^{-1} isocapronic acid). The mixtures were stored at -20 °C until analysis.

For NH_3-N analysis, the frozen samples were thawed, followed by centrifugation at 14000 × g for 10 min. The colorimetric method, described by Scheiner (1976) was used to determine NH_3-N at 623 nm using a spectrophotometer (Evaluation 201; Thermo Fisher Scientific, Waltham, USA). To determine VFA concentration, the thawed samples were centrifuged at 14000 × g for 5 min and the supernatant was used to measure the concentration of VFA by a gas chromatography (Trace GC; Interscience, Milan, Italy) with detection by a flame ionization, as described by Pellikaan *et al*. (2011), using hydrogen as the carrier gas instead of helium.

The amount of base used to increase the pH to neutral (pH 7) was determined in 30 ml of the stomacher solution. A Titrando machine (in conjunction with tiamo software) consist of 907 Titrando, 800 Dosino and 801 Stirrer (Metrohm AG, Herisau, Swizerland) was used to determine the amount of NaOH required to increase the pH to 7 by titrating with 0.1 mol l^{-1} NaOH (Titrisol sodium hydroxide solution; Merck, Darmstadt, Germany). Data were expressed as mmol NaOH required to change the initial pH to 7 per kg DM of the original stored sample.

2.2.4 *In vitro* gas production

In vitro gas production was performed as described by Cone *et al*. (1996). In brief, rumen fluid was collected from three lactating, rumen fistulated cows fed *ad libitum* corn silage and grass silage. The strained rumen fluid was filtered through two layers of cheese cloth and mixed with a mineral-buffer solution. All procedures were conducted under continuous flushing with CO_2. Samples were incubated with the buffered rumen fluid for 72 h and the gas production was automatically recorded. Gas productions was corrected for blank gas production (i.e. gas production in buffered rumen fluid without sample), to allow for fermentation of residual organic matter (OM) in the rumen fluid.

2.2.5 Statistical analysis

In Experiment 1, chemical composition and gas production of each fungal treatment were subjected to the general linear model in SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA). The model was:

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

where Y_{ij} is observation *j* in treatment *i*, μ is the overall mean, α_i is the fix effect of time, ω_{ij} is the random error. Multiple comparisons using Tukey's significant test with $\alpha = 0.05$ in the LSMEANS statement were used to determine significance between treatments.
In Experiment 2, independent sample *t*-tests in SAS, version 9.3 were used to compare the difference between 0 and 64 days of storage.

2.3 Results and discussion

2.3.1 Fungal fermentation of wheat straw

2.3.1.1 pH change of wheat straw after fungal treatment

After autoclaving, the pH of the WS decreased from 5.75 to 5.17 (Fig. 2.1). The pH of the AWS treated with *C. subvermispora* showed an increase from week 0 (5.14) to 1 (5.92), and then gradually decreased to 3.58 after 8 weeks of incubation. The *L. edodes* treated straw showed a slight decrease in pH during the first week, followed by a more pronounced decrease to 4.26 until 5 weeks of incubation, after which the pH remained relatively stable. Apparently, both fungi produced organic acids during the solid state fermentation. Zadražil (1977) reported a decrease in pH of WS after treatment with the basidiomycetes *Pleurotus cornucopiae*, *Pleurotus* sp. Florida, *Agrocybe aegerita* and *Stropharia rugoso-annulata*. In that study, a slight initial increase in pH was also observed, which then remained stable or was decreased by some fungi. A decrease in pH

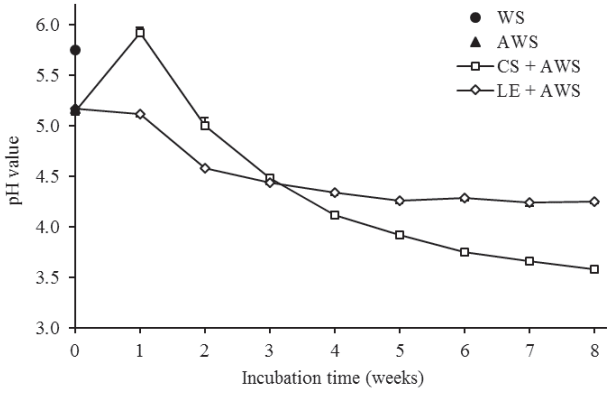

Fig. 2.1. pH value of wheat straw (WS), autoclaved wheat straw (AWS), *Ceriporiopsis subvermispora* treated AWS (CS + AWS) and *Lentinula edodes* treated AWS (LE + AWS) for 0 to 8 weeks (week 0 represents AWS inoculated with spawn without incubation). Error bars indicate the standard deviation.

by the fungi *Dichomitus squalens*, *Trametes ochracea*, and *Trametes versicolor* on wood chips was also reported by Mäkelä *et al*. (2002). Although no data on pH change in WS treated with *C. subvermispora* have been reported, many studies have shown that *C. subvermispora* produces organic acids during growth and colonization of a substrate (Galkin *et al*., 1998; Urzúa *et al*., 1998; Mäkelä *et al*., 2002). Particular acids involved in lignin degradation produced by this fungus are ceriporic acids, which are acids with an itaconic core and different lengths of the alkyl side chains (Amirta *et al*., 2003; Nishimura *et al*., 2012). Hermann *et al*. (2013) reported a similar pH change for the growth of *L. edodes* on sawdust. Although no in depth analyses of the acid production have been conducted for *L. edodes* as far as we know, this fungus is known to produce oxalic acids (Kwak *et al*., 2016).

Acids produced by fungi have diverse functions. Apart from their role in anabolic processes and cellular physiology (Gadd, 1999), acids are excreted to lower the environmental pH to inhibit the growth of competitors allowing fungi to dominate rapidly as the major microorganism (Magnuson and Lasure, 2004). In addition, some organic acids, such as oxalic acid, also have a vital role in lignin degradation (Kuan and Tien, 1993; Urzúa *et al*., 1998; Mäkelä *et al*., 2002). The lowering of pH by fungi during colonization of WS might also have a preservative effect, which might be beneficial for the storage of treated material for use as a feed ingredient.

2.3.1.2 Chemical composition

The changes in the chemical composition during solid state fermentation with *C. subvermispora* and *L. edodes* are shown in Table 2.1 and 2.2. The difference in the chemical composition at week 0 of two inoculated fungi was most likely a result of variation in the WS that we used for each fungal inoculation, even though the straw was collected from the same batch. The hemicellulose and lignin content decreased during the 8 weeks of incubation for both fungi. As a consequence, a significant increase in cellulose, CP and ash content was observed. The increase in ash content indicates the loss of OM during the fungal treatment. Fungi convert 40-50% of the carbon in carbohydrates into carbon dioxide and this is likely the main cause of OM losses (Fernandez and Cadisch, 2003). The increase in CP is thus likely not an absolute increase but an enrichment as a

Table 2.1. Chemical composition of *Ceriporiopsis subvermispora* treated autoclaved wheat straw from 0 to 8 weeks.

Time (Weeks)	Chemical composition				
	Ash	Crude protein	Cellulose	Hemicellulose	Lignin
0	38.8^{d}	27.8^{d}	467.0^{c}	298.5^{a}	76.9^{ab}
1	38.8^{d}	27.3^{d}	473.6^{bc}	296.9^{a}	81.4^{a}
2	38.8^{d}	29.1^{cd}	465.4^{c}	295.0^{a}	79.4^{ab}
3	39.7^{d}	31.0^{bcd}	466.4^{c}	270.5^{b}	74.0^{b}
4	40.7^{c}	31.9^{abc}	466.6^{c}	248.3^{c}	59.6^{c}
5	40.8^{c}	32.6^{abc}	472.0^{bc}	224.2^{d}	45.7^{d}
6	42.4^{b}	33.5^{ab}	471.6^{bc}	200.3^{e}	36.0^{e}
7	42.7^{b}	34.4^{ab}	484.7^{ab}	166.7^{f}	26.8^{f}
8	43.9^{a}	35.0^{a}	494.2^{a}	145.9^{g}	22.0^{f}
RMSE	0.34	1.32	5.02	4.43	2.28
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Ash, g kg^{-1} dry matter; crude protein, cellulose, hemicellulose and lignin, g kg^{-1} organic matter.
RMSE, root mean square error.
$^{a\text{-}f}$ Values within a column with different superscripts are significantly different ($P < 0.05$).

Table 2.2. Chemical composition of *Lentinula edodes* treated autoclaved wheat straw from 0 to 8 weeks.

Time (Weeks)	Chemical composition				
	Ash	Crude protein	Cellulose	Hemicellulose	Lignin
0	54.0^{d}	32.8^{d}	470.6^{c}	299.4^{a}	79.1^{ab}
1	56.1^{cd}	34.3^{cd}	470.7^{c}	293.9^{ab}	82.4^{a}
2	58.4^{c}	36.0^{bc}	485.7^{ab}	279.7^{bc}	83.6^{a}
3	61.2^{b}	36.0^{bc}	489.8^{ab}	268.3^{cd}	81.1^{ab}
4	61.7^{ab}	36.1^{bc}	484.9^{b}	262.2^{d}	74.1^{b}
5	62.3^{ab}	38.4^{ab}	494.5^{ab}	240.7^{e}	59.3^{c}
6	63.8^{a}	39.1^{a}	488.9^{ab}	235.2^{ef}	57.1^{cd}
7	63.7^{a}	37.9^{ab}	498.3^{a}	220.2^{fg}	49.2^{de}
8	64.1^{a}	38.9^{a}	496.6^{ab}	215.5^{g}	48.6^{e}
RMSE	0.89	0.95	4.40	5.71	2.79
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Ash, g kg^{-1} dry matter; crude protein, cellulose, hemicellulose and lignin, g kg^{-1} organic matter.
RMSE, root mean square error.
$^{a\text{-}f}$ Values within a column with different superscripts are significantly different ($P < 0.05$).

result of the loss of carbon. This was indicated by the results of a study by Van Kuijk *et al*. (2015b), showing that the CP concentration significantly increased in *L. edodes* treated regular WS from 0 to 12 weeks of incubation, whereas the absolute amount of CP did not change. Similar results have been obtained by our research group for both *C. subvermispora* and *L. edodes* treated organic WS (Mao L *et al*., unpublished). In addition, CP will be an overestimation of protein because part of the nitrogen will be used to generate mycelial biomass. A substantial part of N is incorporated in chitin in the fungal cell wall and thus not as protein. Changes in the chemical composition of WS after treatment with *C. subvermispora* or *L. edodes* have already been reported (Tuyen *et al*., 2012; Van Kuijk *et al*., 2015b), and are in line with the present study.

2.3.1.3 *In vitro* gas production

The IVGP simulates fermentation in the rumen, because there is a linear relationship between gas production and OM degradation (Cone *et al*., 1996). The total gas production after 72 h of incubation caused by fermentation of WS treated with *C. subvermispora* and *L. edodes* for different weeks is shown in Fig. 2.2. The 72 h gas production with untreated WS and AWS was 188.8 and 214.9 ml g^{-1} OM, respectively. Because autoclaving of WS

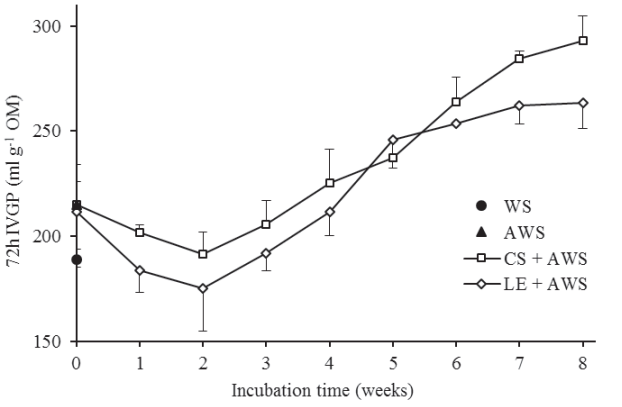

Fig. 2.2. Total gas production after 72 h of wheat straw (WS), autoclaved wheat straw (AWS), *Ceriporiopsis subvermispora* treated AWS (CS + AWS) and *Lentinula edodes* treated AWS (LE + AWS) for 0 to 8 weeks (week 0 represents AWS inoculated with spawn without incubation). Error bars represent the standard deviation.

mimics a thermal treatment, it will increase the enzymatic digestibility (Cybulska *et al*., 2013) and thus might have a positive effect on the IVGP. The gas production of the AWS treated with *C. subvermispora* increased to 292.8 ml g^{-1} OM, and that treated with *L. edodes* increased to 263.7 ml g^{-1} OM at 8 weeks of incubation. The gas production of treated WS decreased until 2 weeks and increased thereafter. The increased gas production after 8 weeks of fungal treatment shows that the nutritive value of the WS increased as a consequence of the decreased lignin content. The decrease in IVGP in the first 2 weeks of fungal treatment indicates a decrease in easily accessible carbohydrates likely consumed first by the fungi before lignin is degraded. Comparable results were reported by Tuyen *et al*. (2012) and Van Kuijk *et al*. (2015b), who showed that gas production of WS increased by treatment of the straw with *C. subvermispora* and *L. edodes* and with a drop in gas production after only 1 or 2 weeks of fungal incubation.

2.3.2 Fungal treated wheat straw stored under anaerobic conditions

2.3.2.1 pH change during storage

To determine whether the fungal treatment has a preservative effect, different parameters were monitored during anaerobic storage of the fungal treated WS. The changes in pH of the untreated and treated WS during the storage process are shown in Fig. 2.3 and 2.4. The pH of the AWS 'as is', with LAB (AWS + LAB) and with a combination of LAB and molasses (AWS + LAB + M) at day 0 of the storage process was 5.14, 5.12 and 5.14,

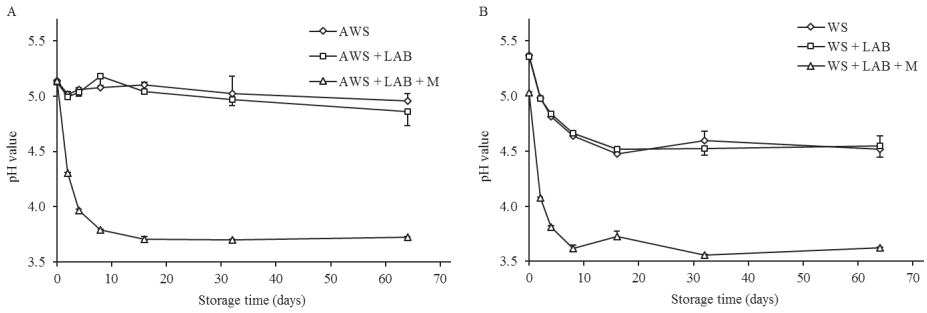

Fig. 2.3. pH value of (A) untreated autoclaved wheat straw (AWS) and (B) wheat straw (WS) after 0, 2, 4, 8, 16, 32 and 64 days of storage. Substrates were stored 'as is', with the addition of lactic acid bacteria (LAB) or with the addition of a combination of LAB and molasses (LAB + M). Error bars represent standard deviation.

respectively (Fig. 2.3A). After 64 days, the pH of AWS and AWS + LAB decreased slightly to 4.95 and 4.86, respectively. By contrast, the pH of AWS + LAB + M showed a rapid decrease to 3.79 on day 8, followed by a period with a stable pH (~3.7) up to 64 days. For the non-AWS, the pH showed a similar pattern during the anaerobic storage as the AWS treatment (Fig. 2.3B), except that, for all treatments, a lower pH was reached. This might be because sterilisation extracts some easily accessible carbohydrates that can be used by the LAB to generate lactic acids. It is unclear, however, why the pH decreases also without the addition of LAB. It is obvious that untreated WS does not contain many nutrients for LAB and that molasses are needed to generate a large pH drop for a stable storage.

The solid state fermentation of straw for 39 days with *C. subvermispora* in Experiment 2 resulted in a lower pH (3.41) compared to the pH (3.58 at 8 weeks) in Experiment 1 and the pH remained stable during the storage process. The addition of LAB and LAB + M hardly influenced the pH (Fig. 2.4A). A similar trend was observed for *L. edodes* (Fig. 2.4B), with a pH of 3.88 after 52 days of aerobic solid state fermentation. A higher value (4.25) was showed in Experiment 1 after 8 weeks of incubation. The addition of LAB + M to *L. edodes* treated AWS resulted in a small decrease in pH, from 4.06 to 3.66 after 8 days of storage, reaching the same value as the *C. subvermispora* treated WS, indicating that this pH is reached by LAB using only the molasses and thus independent of the type

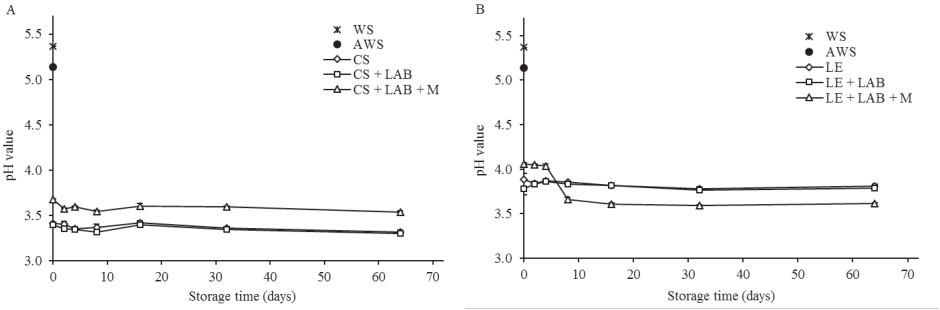

Fig. 2.4. pH value of autoclaved wheat straw treated with (A) *Ceriporiopsis subvermispora* (CS) and (B) *Lentinula edodes* (LE) after 0, 2, 4, 8, 16, 32 and 64 days of storage. Substrates were stored 'as is', with the addition of lactic acid bacteria (LAB) or with the addition of a combination of LAB and molasses (LAB + M). Treatment of AWS and WS are described in Fig. 2.3. Error bars represent standard deviation.

of fungal treated WS. These results show that the decrease in pH by both fungi is sufficient to conserve fungal treated WS under anaerobic conditions for a prolonged period without the need for additives. Studies on the combination of solid state fermentation and ensiling process are rare. Yang *et al*. (2001) used *Penicillium decumbens* as inoculate with a 9:1 ratio of corn straw and wheat bran. After solid state fermentation, the pH reached of 6.3 and addition of LAB and molasses was required to reach a pH drop to 4.5 within one day. Another example of a combination of an ensiling process and a fungal treatment was published by Thomsen *et al*. (2016), who ensiled WS for 4 weeks by adding *L. buchneri* and xylose as a carbon source. The ensiling had a positive effect on the enzymatic degradation of WS and on ethanol production. However, a significant positive effect was only reached if the ensiled material was washed with water before fungal inoculation.

2.3.2.2 Chemical composition and gas production of fungal treated wheat straw during storage

The overall chemical composition of untreated and fungal treated WS did not change much during the 64 days of anaerobic preservation (Table 2.3-2.6). Significant, but small changes were observed in some cases for lignin, cellulose and hemicellulose. However, these changes in chemical composition were not systematic.

The total IVGP did also not change significantly during anaerobic storage. Only in the case of AWS treated with *L. edodes* and with added *L. plantarum*, total gas production was significantly lower by 8.0% after 64 days of preservation compared to 0 days of preservation. Although, for AWS treated with *L. edodes*, a 11.2% decrease was observed. These data show that *C. subvermispora* treated WS can retain its fermentation characteristics as a result of the unchanged fibre composition and its availability for the rumen microbiota. Small changes were observed in IVGP of *L. edodes* treated WS and changes in fibre composition with storage, indicating some degradation. Therefore, from the perspective of chemical composition and gas production, *C. subvermispora* and *L. edodes* treated WS are well preserved anaerobically, either with or without adding LAB and molasses.

Table 2.3. Chemical composition, *in vitro* gas production (IVGP) and silage characteristics of wheat straw (WS) stored anaerobically 'as is', with the addition of lactic acid bacteria (LAB) or with the addition of a combination of LAB and molasses (LAB + M) for 0 (T0) and 64 (T64) days.

Treatment	WS 'as is'		WS + LAB		WS + LAB + M	
	WS-T0	WS-T64	WSL-T0	WSL-T64	WSLM-T0	WSLM-T64
Ash (g kg^{-1} DM)	25.8	22.7*	25.6	22.5*	36.8	38.0*
Crude protein (g kg^{-1} OM)	23.2	24.5	27.6	24.1*	31.9	32.9
Cellulose (g kg^{-1} OM)	494.3	499.3	495.5	500.3	455.4	461.2
Hemicellulose (g kg^{-1} OM)	325.0	298.0*	320.0	311.9	291.7	297.5
Lignin (g kg^{-1} OM)	81.4	74.9*	80.2	74.8*	72.7	71.9
IVGP (ml g^{-1} OM)	192.4	184.8	182.8	177.9	194.2	174.9
Acetic acid (g kg^{-1} DM)	5.70	13.56*	5.41	12.78	6.59	8.35*
NH_3-N (g kg^{-1} total N)	61.2	163.6	48.6	263.2*	64.0	95.5*
NaOH amount (mmol kg^{-1} DM)$^{+}$	33.3	213.5*	34.7	204.9*	55.9	518.9*

DM, dry matter; OM, organic matter; N, nitrogen; N-NH_3, ammonia N.

* Significantly different ($P < 0.05$) from corresponding value at day 0.

$^{+}$ Amount of 0.1 mol l^{-1} NaOH required to increase the pH to 7.

Table 2.4. Chemical composition, *in vitro* gas production (IVGP) and silage characteristics of autoclaved wheat straw (AWS) stored anaerobically 'as is', with the addition of lactic acid bacteria (LAB) or with the addition of a combination of LAB and molasses (LAB + M) for 0 (T0) and 64 (T64) days.

Treatment	AWS 'as is'		AWS + LAB		AWS + LAB + M	
	AWS-T0	AWS-T64	AWSL-T0	AWSL-T64	AWSLM-T0	AWSLM-T64
Ash (g kg^{-1} DM)	24.3	25.0*	24.1	25.0*	35.6	37.7
Crude protein (g kg^{-1} OM)	27.9	28.5	27.8	27.3	31.5	35.9
Cellulose (g kg^{-1} OM)	493.7	491.5	495.4	493.2	447.2	471.8
Hemicellulose (g kg^{-1} OM)	311.9	326.4*	306.8	322.8	284.9	272.7
Lignin (g kg^{-1} OM)	99.1	82.3*	94.3	87.9*	83.8	80.4
IVGP (ml g^{-1} OM)	200.0	192.2	199.6	186.3	212.1	187.0
Acetic acid (g kg^{-1} DM)	6.47	6.33	6.16	7.58*	5.66	7.63*
NH_3-N (g kg^{-1} total N)	85.6	88.6	84.9	86.7	82.1	50.3
NaOH amount (mmol kg^{-1} DM)$^{+}$	41.3	56.0*	41.4	72.7*	55.9	379.5*

DM, dry matter; OM, organic matter; N, nitrogen; N-NH_3, ammonia N.

* Significantly different ($P < 0.05$) from corresponding value at day 0.

$^{+}$ Amount of 0.1 mol l^{-1} NaOH required to increase the pH to 7.

Table 2.5. Chemical composition, *in vitro* gas production (IVGP) and silage characteristics of *Ceriporiopsis subvermispora* treated autoclaved wheat straw (CS, solid state fermentation for 39 days) stored anaerobically 'as is', with the addition of lactic acid bacteria (LAB) or with the addition of a combination of LAB and molasses (LAB + M) for 0 (T0) and 64 (T64) days.

Treatment	CS 'as is'		CS + LAB		CS + LAB + M	
	CS-T0	CS-T64	CSL-T0	CSL-T64	CSLM-T0	CSLM-T64
Ash (g kg^{-1} DM)	24.3	25.2*	24.9	24.9	35.8	36.2
Crude protein (g kg^{-1} OM)	36.3	37.2	35.3	37.8	39.9	41.4
Cellulose (g kg^{-1} OM)	475.2	473.8	475.9	472.4	435.2	427.3
Hemicellulose (g kg^{-1} OM)	165.7	173.4	184.2	179.7	161.3	168.3
Lignin (g kg^{-1} OM)	40.9	35.9*	29.5	34.8	28.6	30.1
IVGP (ml g^{-1} OM)	269.6	268.5	264.5	253.9	263.1	273.5
Acetic acid (g kg^{-1} DM)	1.02	4.93*	0.59	5.35*	0.67	3.45*
NH_3-N (g kg^{-1} total N)	1.80	23.6*	1.72	23.6*	10.5	32.3*
NaOH amount (mmol kg^{-1} DM)$^{+}$	157.3	253.1*	156.0	251.4*	175.4	264.2*

DM, dry matter; OM, organic matter; N, nitrogen; N-NH_3, ammonia N.

* Significantly different ($P < 0.05$) from corresponding value at day 0.

$^{+}$ Amount of 0.1 mol l^{-1} NaOH required to increase the pH to 7.

Table 2.6. Chemical composition, *in vitro* gas production (IVGP) and silage characteristics of *Lentinula edodes* treated autoclaved wheat straw (LE, solid state fermentation for 52 days) stored anaerobically 'as is', with the addition of lactic acid bacteria (LAB) or with the addition of a combination of LAB and molasses (LAB + M) for 0 (T0) and 64 (T64) days.

Treatment	LE 'as is'		LE + LAB		LE + LAB + M	
	LE-T0	LE-T64	LEL-T0	LEL-T64	LELM-T0	LELM-T64
Ash (g kg^{-1} DM)	25.3	25.3	25.4	25.7	37.7	38.5*
Crude protein (g kg^{-1} OM)	38.7	39.3	41.4	42.0	46.9	47.0
Cellulose (g kg^{-1} OM)	510.0	498.1*	507.5	499.3	456.7	443.8*
Hemicellulose (g kg^{-1} OM)	181.4	182.3	178.9	166.6	166.8	145.1*
Lignin (g kg^{-1} OM)	42.5	42.3	38.0	40.8	33.6	33.6
IVGP (ml g^{-1} OM)	267.6	237.6	267.1	245.7*	251.4	241.9
Acetic acid (g kg^{-1} DM)	1.71	3.18	0.92	3.15*	1.36	4.32*
NH_3-N (g kg^{-1} total N)	5.06	25.7*	4.32	24.5*	7.94	31.9*
NaOH amount (mmol kg^{-1} DM)$^{+}$	157.5	222.7*	161.7	235.5*	175.0	442.9*

DM, dry matter; OM, organic matter; N, nitrogen; N-NH_3, ammonia N.

* Significantly different ($P < 0.05$) from corresponding value at day 0.

$^{+}$ Amount of 0.1 mol l^{-1} NaOH required to increase the pH to 7.

2.3.2.3 Concentration of VFA and NH_3-N and amount of NaOH required

After the storage period of 64 days, acetic acid was detected in all samples of WS, AWS, and fungal treated WS (Table 2.3, 2.4, 2.5 and 2.6). Only minor amounts of butyric acid, propionic acid, isobutyric acid, isovaleric acid and valeric acid were detected after the 64 days of preservation (data not shown). The treatment with both *C. subvermispora* (Table 2.5) and *L. edodes* (Table 2.6) decreased the concentration of acetic acid, compared to untreated WS, although the concentration recovered to some extent during the storage period. The same pattern was seen for the NH_3-N concentration. During the ensiling of grass and maize, acetic acid and NH_3-N are produced by the activity of microorganisms. Although the low pH generated by the fungi will inhibit most microbial growth, it is possible that some microorganisms can still grow and cause changes in NH_3-N and acetic acid concentrations.

The buffer capacity of some substrates, such as dried WS, was relatively low, which means that minor amounts of acid could cause a rapid decrease in pH. Therefore, whether the acids produced by the fungi are sufficient for the preservation of the fungal treated WS or not is unknown. If the preservation of WS by fungi is mainly caused by the decrease in pH, it is important to estimate the amount and strength of the acids formed. The latter can be achieved by measuring the amount of alkali (NaOH) required to increase the pH to 7.0. For neutralizing the fungal treated straw (*C. subvermispora*: 157.3 mmol kg^{-1} DM, *L. edodes*: 157.5 mmol kg^{-1} DM), approximately four times the amount of NaOH was needed compared to the untreated AWS (41.3 mmol kg^{-1} DM). During the 64 days of anaerobic storage, however, the amount of NaOH needed for neutralisation also increased. This correlates with the increase in acetic acid during the storage period. We assume white-rot fungi to be metabolically inactive during the anaerobic storage period, hence, the increase in NaOH consumption must have another, previously unknown reason. Although the amount of acids increased during storage, this was not detected by a change in pH. The latter can be explained by the fact that pH is expressed on a logarithmic scale and small changes in pH can lead to a significant increase in the alkali required for neutralisation.

2.4 Conclusions

Both *C. subvermispora* and *L. edodes* improved the fermentability of wheat straw over an 8 week period as determined by the IVGP. Both fungi substantially decreased the pH

during solid state fermentation. The pH decrease < 4.3 by *C. subvermispora* and *L. edodes* appears to be effective for stabilising wheat straw, as indicated by the unchanged fibre composition and retained increased fermentability measured with the IVGP method. Fungal treated wheat straw can be conserved under anaerobic conditions, without adding lactic acid bacteria and molasses. The latter is highly desirable with respect to the practical use of this technology as a feed resource for ruminants.

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Chapter 3

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

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Abstract

To understand the effect of fungal treatment on quality of feed for ruminants, the compounds formed from *Ceriporiopsis subvermispora* and *Lentinula edodes* cultured on wheat straw for 0 to 8 weeks were investigated using GC-MS and LC-MS. Changes in pH, ergosterol content and fibre composition were also evaluated. With increases in mycelium content, significant decreases in absolute amount of hemicellulose and ADL and, to a lesser extent, cellulose were observed during both fungal treatments, with a dry matter loss of 15.9% caused by *C. subvermispora* and 17.5% by *L. edodes* after 8 weeks. The fungi acidified the wheat straw mainly within 4 weeks, when largest changes in profile of formed compounds occurred. Diverse compounds, including organic acids and soluble sugars accumulated or decreased by the *C. subvermispora* and *L. edodes* treatments. No known mycotoxins were found in the two fungal cultures. Those results give important information for practical application since the produced compounds might affect animal acceptance and performance.

Key words

Ceriporiopsis subvermispora; *Lentinula edodes*; fibre composition; mycotoxins; organic acids; sugars.

3.1 Introduction

White-rot fungi are considered as the most dominant microbiota in decaying lignified biomass. These fungi can be used to increase the availability of structural polysaccharides through breakage of C-C and ether linkages in lignin, decrease the cellulose crystallinity and break bonds between polysaccharides and lignin (Dilokpimol *et al*., 2016) by producing a set of peroxidases and auxiliary enzymes. The degradation capacity of white-rot fungi depends on the fungal species/strain, substrate, incubation conditions and selected additives (Tuyen *et al*., 2012, 2013; Van Kuijk *et al*., 2016a; Nayan *et al*., 2018). Some fungi are classified as non-selective fungi, which attack lignin as well as the polysaccharides cellulose and hemicellulose. Selective fungi degrade lignin and hemicellulose but little cellulose. This group includes *Ceriporiopsis subvermispora* and *Lentinula edodes*, which have been compared to a number of other white-rot fungi (Tuyen *et al*., 2012) and shown to be the best to increase in fermentability by rumen microbes of wheat straw (WS).

Stability and safety are important criteria for the use of fungal treated substrates as animal feed. A wide variety of (secondary) metabolites are formed by white-rot fungi or compounds are released from lignocellulosic biomass during bioconversion. Both can affect the nutritional value and acceptance of the treated biomass by ruminant animals. Previous studies indicate that *C. subvermispora* and *L. edodes* are able to produce a series of organic acids and hence acidify the substrate (Mäkelä *et al*., 2002; Kwak *et al*., 2016), as well as release free sugars during colonization on lignocellulosic biomass. In addition, *C. subvermispora* excretes different organic acids compared to most other white-rot fungi, i.e. ceriporic acids (Enoki *et al*., 2002; Amirta *et al*., 2003; Nishimura *et al*., 2008; Nishimura *et al*., 2012a and b), which are important for cell wall degradation. Different types of organic acids are produced by these fungi in the process of degradation of lignin and an analysis of these and other compounds will enhance our understanding of lignin degradation. Moreover, these analyses can provide more insight in quality and safety of fungal treated lignocellulose as an animal feedstuff.

Mycotoxins are a series of compounds that could have adverse effects on animals and humans. Some filamentous fungi are reported to produce mycotoxins as secondary metabolites, whereas other white-rot fungi are shown to have detoxification capabilities (Wang *et al*., 2011; Yehia, 2014). *L. edodes* is an edible and medicinal fungus that has been widely used in the cultivation of edible mushrooms and is considered safe for

consumption. *C. subvermispora*, however, is a crust fungus which does not produce harvestable mushrooms for consumption and has hardly been used in feeding studies.
This study measured the changes in the selected organic compounds during 8 weeks of treatment by *C. subvermispora* and *L. edodes* on WS using the gas chromatography mass spectrometry (GC-MS) technique, a good and reliable method to estimate major organic compounds. Using liquid chromatography mass spectrometry (LC-MS), four ceriporic acids (A, B, C and G) were determined from *C. subvermispora* treated WS. Analysis for the presence of 34 common mycotoxins in *C. subvermispora* and *L. edodes* treated WS was also conducted.

3.2 Materials and methods

3.2.1 Fungal strain and spawn preparation

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

3.2.2 Fungal inoculation and incubation

Dry organic WS was mechanically chopped to 0.5-2 cm with a chop machine (Pierret Industries, Corbion, Belgium), filled in monofilament net bags (50 × 80 cm, pore size: 1.5 × 1.5 mm) and immersed in tap water for three days. The straw was drained for 5 h and mixed by hand. The fungal treatments were carried out in 1.2 l plastic boxes (TP1200 + TPD1200; Combiness, Nevele, Belgium), with 240 g wet WS in each box, before autoclaving at 121 °C for 1 h. All boxes were left overnight for further fungal inoculation. The autoclaved WS was inoculated with 6 g of each fungal spawn, and the boxes were then maintained at 25 °C in a climate controlled room for 8 weeks. Three boxes of each

fungal treated WS were weighed and collected at week 0, 1, 2, 4, 6 and 8 weeks of incubation for further analysis.

3.2.3 Sample analysis

3.2.3.1 Chemical composition and pH

The substrate in the boxes were freeze dried and milled to pass a 1 mm sieve (100 AN; Peppink, Olst, The Netherlands). The dry matter (DM) content was determined at 103 °C for 4 h according to the methods described in ISO 6496 (1999), the dried samples were then incinerated in a muffle furnace at 550 °C for 3 h to determine the ash content (ISO 5984, 2002). The nitrogen (N) content was determined according to ISO 5983 (2005). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined as described by Van Soest *et al*. (1991), using an automatic Ankom fiber analyzer (A2000I; ANKOM Technology, Macedon, NY, USA). Cellulose was calculated as the difference between ADF and ADL and hemicellulose as the difference between NDF and ADF. The loss of DM and absolute amount of the cell wall components was calculated, based on the weight change of the fungal treatment from the beginning to the end of the incubation. The pH of the dried and ground samples was measured in a titrando machine (Metrohm, Herisau, Swizerland) after adding 30 ml demineralised water to 1 g of sample and mixing for 30 min at 230 rpm in a shaking machine.

3.2.3.2 Ergosterol measurement

The ergosterol content of the freeze dried samples was determined as described by Niemenmaa *et al*. (2008). Briefly, approximately 0.2 g sample was weighed into a glass tube to which 3 ml of 10% KOH in methanol solution was added. The suspension was mixed 10 min at 230 rpm and saponified at 80 °C for 60 min. As an internal standard, 20 µl of cholecalciferol (vitamin D_3, 0.5 µg μl^{-1}) was added, followed by adding 1 ml of H_2O and 2 ml of hexane after the suspension was cooled down to room temperature. The hexane phase was transferred to a new glass tube after centrifuging of the suspension at 4000 rpm for 10 min, and the extraction procedure with water and hexane was repeated. The collected hexane phase was then evaporated in an vacuum evaporator at 30 °C for 60 min. The residue was dissolved in 1 ml of methanol and vortexed for five sec. The solution was filtered and transferred to a glass vial with insert for HPLC analysis

(Alliance HPLC system, Milford, USA) which consisted of a Waters HPLC-PDA system and a Phenomex aqua 5 µm C18 column (250 × 4.6 mm). The solvent was 90% methanol and 10% (1:1) 2-propanol/hexane, with a flow rate of 0.5 ml/min and measurement of ergosterol at 280 nm.

3.2.3.3 Mycotoxin analysis

The mycotoxin content was analysed using a validated and accredited LC-MS/MS-based analytical method at the Dutch National Reference Laboratory RIKILT (Wageningen University & Research, Wageningen, The Netherlands). Prior to the analysis, approximately 0.9 g of the substrate was weighed in a 50 ml plastic tube and 6 ml of water, 10 ml of extraction solvent (acetonitrile/acetic acid 990:10 (v/v)), and 25 µl of ^{13}C-caffeine internal standard (IS) were added. The procedure was adjusted according to the availability of the substrates. The suspension was shaken manually and placed in a extracting device (Heidolph Reax 2, Schwabach, Germany) for 30 min. Four g of magnesium sulphate was added, the tube vortexed for one min and centrifuged at 3000 rpm for 10 min. Two hundred µl of extract was transferred into polypropylene vials (Whatmann syringeless filter device) and diluted with 200 µl water, capped and shaken in a vortex mixer for approximately 3 sec. After 30-60 min in the refrigerator (+ 4°C), the vials were closed with a pressing device (Six Position Compression; Whatman, 's-Hertogenbosch, The Netherlands) and stored at + 4 °C until analysed by an LC-MS/MS (Waters Acquity, Etten-Leur, the Netherlands; AB SCIEX QTRAP® 6500, Applied Biosystems, Nieuwekerk aan de IJssel, the Netherlands) with a Restek Ultra Aqueous C18 3 µm 100 × 2.1 mm UPLC column (Restek Corporation, Bellefonte, USA) and a sample injection volume of 5 µl. The gradient was achieved using two different eluents: Eluent A consisted of 1 mM ammonium formate and 1% (v/v) formic acid in water; Eluent B consisted of 1 mM ammonium formate and 1% (v/v) formic acid in a mix of methanol and water 95:5 (v/v). Eluent C consisted of 5 mM ammonium acetate and 0.1% (v/v) acetic acid in water; Eluent D consisted of 5 mM ammonium acetate and 0.1% (v/v) acetic acid in a mix of methanol and water 95:5 (v/v). Eluent A and B were used when analysing mycotoxins in the positive mode (ESI+), eluent C and D were used when analysing mycotoxins in the negative mode (ESI-). Each mycotoxin was identified by its retention time and the peak area ratio between two transitions (Supplementary, Table S3.1). For nitropropionic acid and moniliformin, however, one transition was available.

Therefore, identification of these mycotoxins was regarded to be tentative. Quantification was performed by a single point standard addition protocol. Validation parameters can be found in supplementary Table S3.1.

3.2.3.4 Gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) profiling

The relative content of various compounds was determined by GC-MS analysis with extraction according to the method described by Lisec *et al*. (2006). Modifications were performed enabling a better extraction of compounds from material. Briefly, approximately 50 mg (48-52 mg) ground sample was extracted in a 2 ml Eppendorf tube with 1.4 ml of 80% pre-cooled methanol (-20 °C), and mixed with 60 µl of ribitol (0.2 mg ml^{-1}) as internal quantitative standard. After shaking in a thermomixer at 950 rpm at 70 °C for 10 min (Vortemp 56; Labnet International Inc., Edison, USA) and centrifuging in a Eppendorf centrifuge for 10 min at top speed (21000 × g), 500 µl of the supernatant was transferred into an new Eppendorf tube, and mixed with 375 µl of chloroform and 750 µl of H_2O for 10 s. The mixture was separated by centrifuging at top speed (21000 × g) in a Eppendorf centrifuge and 100 µl of the methanol-water phase was transferred into the insert glass vial. All the extracts were dried by a vacuum centrifugation.

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

The determination of ceriporic acids A, B, C and G was conducted by target analysis LC-MS. The compounds were extracted by the protocol as described by De Vos *et al*. (2007). In short, approximate 100 mg (97-103 mg) sample powder was weighed and extracted with 1.5 ml of 75% methanol and 0.1% of formic acid. The suspension was vortexed (10 s) and then sonicated for 10 min. The phase was separated by centrifuging for 10 min in an Eppendorf centrifuge (16000 × g), and 180 µl of the supernatant was collected to an insert glass vial for further LC-MS analysis. Chromatographic separation, mass detection methods and the equipment is described by Mokochinski *et al*. (2018). The mass spectra

of ceriporic acid A, B and C were identified to that reported by Amirta *et al*. (2003) and Van Kuijk *et al*. (2017), while the mass spectra of ceriporic acid G was identified according to Nishimura *et al*. (2012b).

3.2.4 Data processing and statistics analysis

The data processing and statistical analysis of the GC-MS and LC-MS analysis were performed according to steps described by Mokochinski *et al*. (2018). The principal components analysis (PCA) was performed using the software Simca (version 14) from Umetrics.

The pH, chemical composition and ergosterol data were analysed with the following generalized linear model (GLM) of SAS 9.3:

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

where Y_{ij} is observation *j* in treatment *i*, μ is the overall mean, α_i is the fix effect of time and ω_{ij} is the random error. Multiple comparisons using Tukey's significant test with $\alpha = 0.05$ was used to determine significance between treatments.

3.3 Results and discussion

3.3.1 pH and mycelium growth of fungal strains

The extracts showed a decrease in pH of the WS after *C. subvermispora* and *L. edodes* treatment for 8 weeks (Fig. 3.1A). *C. subvermispora* reduced the pH of the autoclaved WS from 4.75 to 3.30, whereas *L. edodes* reduced the pH from 4.74 to 3.92. A number of studies reported the acidification and organic acid production by white-rot fungi in a liquid or solid culture environment (Mäkelä *et al*., 2002; Kwak *et al*., 2016). Since the analysis was done on freeze dried samples, this indicates that the pH decrease is mainly caused by non-volatile organic acids. In a previous study (Mao *et al*., 2018), pH was measured in fresh samples and a similar pH profile was observed during fungal incubation. The only difference was that in fresh samples an initial increase in pH was seen in the first week with *C. subvermispora* which might have been caused by volatile acids. This increase was also observed by Zadražil (1977) with other white-rot fungi.

Ergosterol is a sterol almost exclusively found in fungal cell membranes, which enables ergosterol as an indicator for fungal biomass (Niemenmaa *et al*., 2008). *C. subvermispora* showed a linear growth in the first 6 weeks (92.2 μg ergosterol g^{-1} dry substrate) of

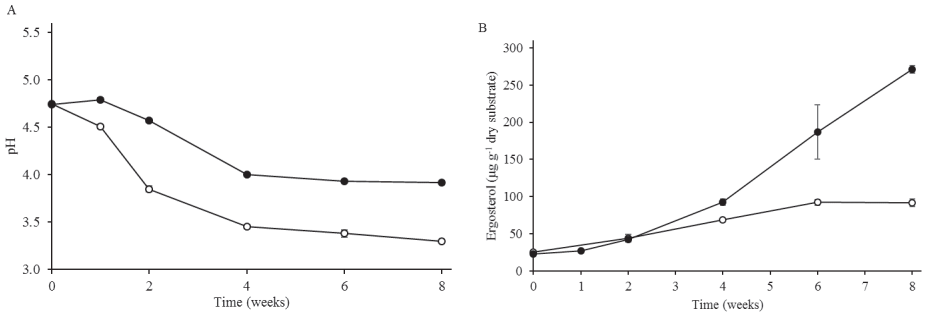

Fig. 3.1. pH (A) and ergosterol content (B) of *Ceriporiopsis subvermispora* (○) and *Lentinula edodes* (●) grown on autoclaved wheat straw for 0, 1, 2, 4, 6 and 8 weeks.

incubation after which the fungal biomass remained the same. *L. edodes* had a lag phase of growth in the first week after which growth increased linearly between 4 and 8 weeks of incubation (270.9 µg ergosterol g^{-1} dry substrates) (Fig. 3.1B). The increased ergosterol content indicates that both *C. subvermispora* and *L. edodes* colonized the WS well but *L. edodes* formed clearly more biomass as could also be seen visually. It is clear that *C. subvermispora* produced more or stronger organic acids since especially in the first two weeks, where both fungi had a similar mycelium content, a greater decrease in pH was seen than in *L. edodes* samples. The pH change for *C. subvermispora* almost coincided with its growth: when the increase in biomass stopped, the pH remained almost unchanged. For *L. edodes*, however, growth continued during the whole incubation period whereas the pH remained almost unchanged after 4 weeks. In an elegant study, Blanchette *et al*. (1997) have shown that even with the presence of single hypha of *C. subvermispora* in a plant cell wall lumen, a clear degradation/alteration of the cell wall is seen. That might indicate that *C. subvermispora* is producing strong or more acids/radicals than other white-rot fungi and in accordance with our comparison between this fungus and *L. edodes*. The latter produces much more biomass but reduces the pH of the medium less than *C. subvermispora* and also shows less lignin degradation (as shown in the next paragraph).

3.3.2 Fibre composition

The absolute amount of ash did not change during the incubation of 8 week with *C. subvermispora* ($P > 0.05$) and *L. edodes* ($P > 0.05$) (Table 3.1), indicating that the

Table 3.1. Absolute amount (g) and dry matter (DM) loss of chemical components of autoclaved wheat straw with culture of *Ceriporiopsis subvermispora* and *Lentinula edodes* from 0 to 8 weeks.

Duration (Week)	*C. subvermispora*						*L. edodes*					
	Ash	Nitrogen	Cellulose	Hemicellulose	ADL	DM loss (%)	Ash	Nitrogen	Cellulose	Hemicellulose	ADL	DM loss (%)
0	2.12	0.173	24.9^{a}	14.8^{a}	3.86^{b}	0.00^{e}	2.11	0.175	24.6^{ab}	14.6^{a}	3.77^{a}	0.00^{e}
1	2.20	0.173	24.4^{ab}	14.7^{a}	4.30^{a}	0.79^{e}	2.12	0.176	25.2^{a}	13.8^{b}	3.50^{b}	0.73^{de}
2	2.11	0.178	24.2^{bc}	12.1^{b}	3.46^{c}	3.57^{d}	2.13	0.180	25.0^{a}	13.6^{b}	3.71^{ab}	1.82^{d}
4	2.13	0.172	23.8^{cd}	9.28^{c}	1.97^{d}	8.70^{c}	2.10	0.172	24.7^{a}	9.87^{c}	2.92^{c}	7.35^{c}
6	2.08	0.172	23.4^{d}	6.05^{d}	1.36^{e}	14.2^{b}	2.10	0.169	24.0^{bc}	8.24^{d}	2.42^{d}	12.4^{b}
8	2.12	0.181	23.2^{d}	5.15^{d}	1.08^{e}	15.9^{a}	2.07	0.169	23.5^{c}	6.89^{e}	2.01^{e}	17.5^{a}
RMSE	0.07	0.01	0.23	0.95	0.10	0.50	0.03	0.01	0.23	0.23	0.09	0.47
p-value	0.535	0.389	< 0.001	< 0.001	< 0.001	< 0.001	0.342	0.380	< 0.001	< 0.001	< 0.001	< 0.001

RMSE, root mean square error.

$^{a\text{-}e}$ Means with different superscripts within a column are significantly different ($P < 0.05$).

gravimetric analysis of the samples were carried out correctly. As expected, the nitrogen content also did not change significantly since nitrogen in WS will be incorporated into fungal biomass. A decrease in the absolute amount of cellulose, hemicellulose and lignin was observed. After 8 weeks of *C. subvermispora* treatment, 6.8% cellulose, 65.2% hemicellulose and 72% ADL reduction was seen, and 15.9% DM loss was observed. For *L. edodes*, 4.5% cellulose, 52.8% hemicellulose, 46.7% ADL reduction was seen and 17.5% DM loss after 8 weeks of incubation. The low mineralization of cellulose was also observed in previous experiments (Tuyen *et al*., 2012; Mao *et al*., 2018; Nayan *et al*., 2018) and is common for selective white-rot fungi (Martínez *et al*., 2011).

The structural carbohydrates and ADL were determined using the detergent fibre analysis methods by Van Soest *et al*. (1991). The method is quick and useful to see trends in changes in cell wall fibres. The method is, however, designed for feed analysis and especially the measurements of carbohydrates can be inaccurate since these are measured as residues after extraction (thus as insoluble components). As a consequence, the reduction in hemicellulose measured here might be for a part due to the solubilisation and not due to consumption of the fungi as has been shown by Nayan *et al*. (2019). The content of cellulose can also be overestimated due to glucan in fungal cell walls (Nayan *et al*., 2019), while ADL does not represent the total lignin content, due to the fact that acid soluble lignin is lost with this method (Godin *et al*., 2014). The detergent fibre method for measuring ADL is, however, a good estimate for the more "recalcitrant" insoluble lignin that correlates well with *in vitro* degradation in rumen fluid (Nayan *et al*., 2019). *C. subvermispora* shows a more rapid reduction in ADL and to a higher extent than *L. edodes*. Previous research has shown that this also leads to a higher *in vitro* digestibility (Tuyen *et al*., 2012; Mao *et al*., 2018; Nayan *et al*., 2018). The cellulose content (measured as ADF-ADL) is slightly reduced by both fungi whereas the hemicellulose content (measured as NDF-ADF) is strongly reduced and, as mentioned previously, at least in part due to solubilisation. This higher reduction in hemicellulose might also be due to a higher degradation of ADL which is covalently bound to hemicellulose (Hatakka and Hammel, 2011). The loss of DM during the 8 weeks of incubation is very similar for both fungi. One would expect that the DM loss for *L. edodes* would be higher since more fungal biomass is formed, assuming that approximately half of the carbon is converted to new fungal biomass and the other half to CO_2. The amount

of fungal biomass formed, however, represents only a small part of the total dry matter (Nayan *et al*., 2019) and might be below the detection level.

3.3.3 Compounds released during fungal treatment

To provide a general impression of changes in compounds in time and between the two fungi, principal component analysis (PCA) was performed, based on all compounds (identified and unknown) detected by the GC-MS analysis. The PCA plot described 52.1% of the total variation in the first two principal components of the GC-MS analysis (Fig. 3.2). The analysis of samples of weeks 0, 4 and 8 were conducted in biological triplicate, the other time points were single measurements. The individual points in each triplicate cluster indicates a high reproducibility of the biological triplicates. A clear distinction is seen between each incubation time point and fungal strain. The difference between week 0 point of *C. subvermispora* and *L. edodes* originates likely from the added spawn at the beginning of the incubation. *L. edodes* is degrading the starch of spawn to a much greater extent than *C. subvermispora* (Van Kuijk *et al*., 2016b). The largest change in compounds

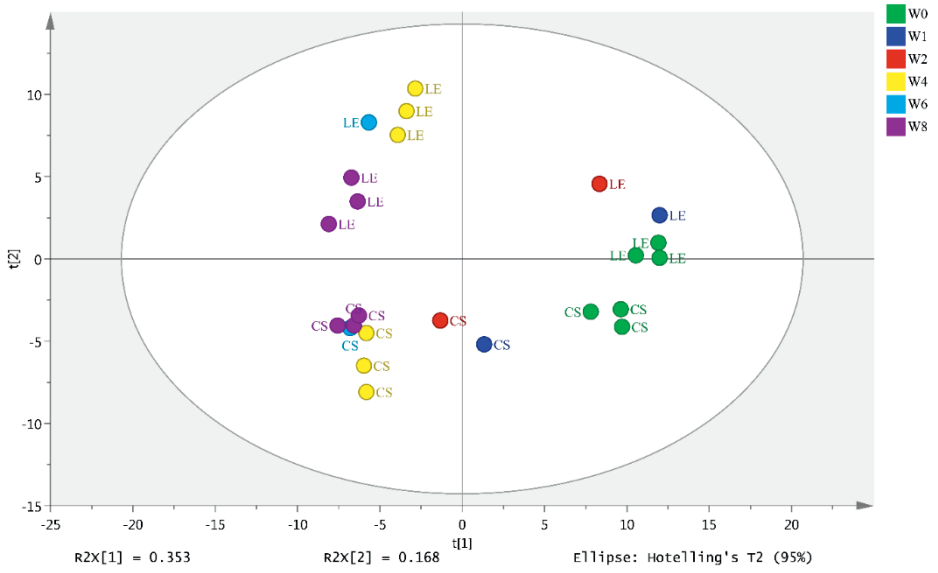

Fig. 3.2. Principal components analysis plots of compounds from GC-MS analysis of autoclaved wheat straw treated with *Ceriporiopsis subvermispora* (CS) and *Lentinula edodes* (LE) for 0, 1, 2, 4, 6 and 8 weeks (W). Week 0, 4 and 8 analyses were performed in biological triplicates, week 1, 2 and 6 were single measurements.

formed from the GC-MS analysis mainly occurs from week 0 to 4 for both fungi in the principle components. Although the measurements for week 1, 2 and 6 are singular, they seem to support the pH and ergosterol data: *C. subvermispora* starts earlier in growth and decreasing the pH and obviously also shows an earlier change in compounds than *L. edodes*.

The formed compounds that could be identified are classified into three groups: organic acids, carbohydrates and others (Table 3.2). Since no analysis was performed on dry wheat straw before soaking in water, we cannot exclude that some organic acids were already present in WS. As expected, oxalic acid accumulated in the *C. subvermispora* and *L. edodes* treatments until 8 weeks. This is a common organic acid produced by white-rot fungi in either solid or liquid culture (Mäkelä *et al*., 2002; Kwak *et al*., 2016). Oxalic acid detected in week 0 originates likely from the colonization of fungi on sorghum grain or partly from straw. High amounts of lactic and succinic acids were present in samples of week 0 of the two fungi and decreased after 8 weeks. These components are likely generated during the three days submersion of WS as under low oxygen conditions, fermentations from microorganisms, such as lactic acid bacteria can occur. Substrates for fermentation under these conditions can be free sugars that are found in WS (Shan *et al*., 2008; Tishler *et al*., 2015). The quick disappearance of these acids indicates metabolisation by the inoculated fungi. Vanillic acid and protocatechuic acids are both breakdown products and different catabolic funnelling pathways involved have been described (Abdelaziz *et al*., 2016). The relative higher concentration of vanillic acid by *C. subvermispora* corresponds to the better degradation of lignin compared to *L. edodes*. Galacturonic acid is the main component of pectin. The clear accumulation in the *L. edodes* treatment and lack of production in the *C. subvermispora* treatment might indicate a different pathway in degradation of pectin in cell walls. Both fungi produced different low molecular weight organic acids that were also previously reported for other white-rot fungi (Liaud *et al*., 2014) and also found in nature in upper soil levels as degradation products by fungi (Strobel, 2001). These acids play an important role in acidifying the environment and creating an advantage for fungi over bacteria. Organic acids, especially carboxylic acid, also play an important role in the degradation of lignin as a chelator (Kishi *et al*., 1994). Both fungi differ in the amount of organic acids produced. Whereas

Table 3.2. Compounds released from autoclaved wheat straw treated with *Ceriporiopsis subvermispora* and *Lentinula edodes* for 0 and 8 weeks.

					C. subvermispora		*L. edodes*	
Type	Centrotype	RIexp	Name	Annotation level*	Week 0	Week 8	Week 0	Week 8
Organic acid	303	1074	Lactic Acid, 2TMS	1	23299	201	26384	76
	514	1088	Glycolic acid, 2TMS	1	4567	1472	4785	1349
	1076	1141	Oxalic acid, 2TMS	1	8050	12496	6159	10628
	2828	1301	Succinic acid, 2TMS	1	65043	1131	61938	1284
	2965	1312	Glyceric acid, 3TMS	2	3176	3039	3201	2627
	3675	1384	Formic acid, 1TMS	2	383	2766	369	146
	3756	1392	Malonic acid, 2TMS	2	244	110	346	66
	4535	1473	Malic acid, 3TMS	1	632	736	615	1574
	5045	1515	Pipecolic acid, 2TMS	2	757	564	601	802
	5362	1543	2,3,4-Trihydroxybutyric acid, 4TMS	2	2109	1628	1870	251
	6103	1623	Tartaric acid, 4TMS	1	0	1794	4	39
	8528	1749	Ribonic acid, 4TMS	1	2576	1749	2542	655
	8758	1765	Vanillic acid, 2TMS	1	1274	4952	891	2871
	9263	1816	Protocatechuic acid, 3TMS	1	7588	375	7522	133
	11840	1924	Galacturonic acid, 5TMS, methyloxime 1	1	11	83	0	1351
Carbohydrate	3340	1345	Dihydroxyacetone, 2TMS	2	111	1575	158	1306
	4761	1489	Threitol, 4TMS	1	5112	471	5167	120
	6170	1627	Arabinose, 4TMS, methyloxime	1	574	2883	577	1748
	6502	1636	Arabinose, 4TMS, methyloxime, metyloxime (isomer 2)	1	12254	2380	12323	475
	7129	1647	Xylose, 4TMS, methyloxime (anti)	1	95827	1366	117970	3293
	7529	1662	Xylose, 4TMS, methyloxime(syn)	1	844	403	902	229

	7959	1704	Arabitol, 5TMS	1	26504	70263	46993	204534
	9832	1857	Fructose, 5TMS, methyloxime (anti)	1	69554	721	65696	1065
	10004	1867	Fructose, 5TMS, methyloxime (syn)	1	56943	310	52301	479
	10263	1877	Mannose, 5TMS, methyloxime (1Z)	1	1433	601	4354	786
	10566	1883	d-Glucose, 5TMS, o-methyloxyme, (1E)	1	9058	996	304169	6486
	11099	1903	d-Glucose, 5TMS, o-methyloxyme, (1Z)	1	2160	177	70759	1181
	11450	1914	Mannitol, 6TMS	1	13039	46814	6202	448761
	12090	1941	Myo-Inositol, 6TMS	1	9566	5935	9766	6812
	13205	2074	Myo-Inositol, 6TMS	2	5758	4733	5650	4221
	14498	2356	Xylobiose, 6TMS, methyloxime 1	1	396	4994	454	2324
	14812	2376	Xylobiose, 6TMS, methyloxime 2	1	104	2034	125	1292
	16191	2545	Sucrose, 8TMS	1	20170	13137	28320	27238
	16763	2625	Maltose, 8TMS, methyloxime (isomer 1)	1	63	0	3512	492
	16978	2631	Trehalose, 8TMS	1	355	46704	341	40563
	17820	2779	Uridine, 3TMS	1	34	282	9	2142
Others	134	1060	2,3-Butanediol, 2TMS	2	30526	117	28980	22
	2308	1257	Glycerol, 3TMS	1	10851	21014	20369	5650

* The annotation was levelled according to the rules described by Sumner *et al*. (2007).

oxalic acid was produced in similar amounts by both fungi, formic and tartaric acids were produced more by *C. subvermispora*, and malic and galacturonic acids more by *L. edodes*. A clear difference in organic acids between the fungi were of course also the ceriporic acids produced only by *C. subvermispora*. Four ceriporic acids (A, B, C and G) were identified by LC-MS with the mass spectral fragmentation from WS cultures with *C. subvermispora* (Fig. 3.3). Those compounds accumulated until four weeks of *C. subvermispora* incubation, and remained stable or slightly decreased thereafter. It is proposed that the lignin degradation is closely related to extracellular lipid peroxidation by *C. subvermispora*, and indeed ceriporic acid G can trigger the lipid peroxidation process catalyzed by manganese peroxide (Nishimura *et al*., 2012b). Since the GC-MS analysis was not a quantitative analysis, we cannot explain which acids correlates best with the pH change during the fungal treatment of WS.

Different water soluble carbohydrates were found by GC-MS analysis in fungal treated WS (Table 3.2). Especially striking are the relatively high concentrations of mono- and disaccharides at the start of the incubation. Xylose and arabinose might originate partly from the non-starch polysaccharides in sorghum grain (Knudsen, 2014; Betts *et al*., 2015) and from WS after autoclaving at week 0. Wheat straw also contains a considerable amount of free sugars (Shan *et al*., 2008; Tishler *et al*., 2015) that might partly explain

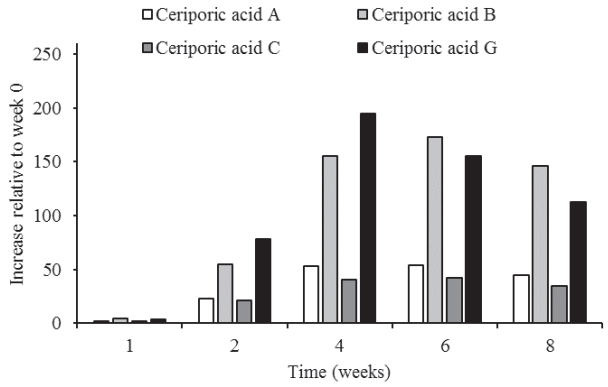

Fig. 3.3. Fold increase (relative to week 0) in ceriporic acid A, B, C and G produced by *Ceriporiopsis subvermispora* during 8 weeks of incubation on autoclaved wheat straw. Week 0, 4 and 8 analyses were performed in biological triplicates, while week 1, 2 and 6 were single measurements.

the amount of sugars at the start of fungal growth. Fructose, arabinose, xylose, mannose and xylobiose might be produced during the submerged period of WS by excreted enzymes derived from fungi present on WS. The high amount of glucose at week 0 for *L. edodes* compared to *C. subvermispora* can be explained by the degradation of starch present in the spawn (Betts *et al*., 2015) added at week 0. *L. edodes* degrades starch much better than *C. subvermispora* (Van Kuijk *et al*., 2016b). For *C. subvermispora*, the xylose (anti) and fructose concentration decreased sharply within first week of incubation (data only shown for week 8). As is often seen with white-rot fungi, first free sugars are metabolized before the more complex cell components are degraded. Glucose concentration, however, decreased after 2 weeks of incubation and was especially high in the first 2 weeks of *L. edodes* treated WS. Remarkable is the accumulation of polyols by both fungi. Polyols are widely distributed in fungi (Rast and Pfyffer, 1989). The relative amount of arabitol and mannitol in *L. edodes* treated WS was approximately 3 and 10 times higher than in *C. subvermispora* and might be explained in part by the differences in the amount of biomass formed. Glycerol, on the other hand, was approximately 4 times higher in *C. subvermispora* than *L. edodes* treated WS. Polyols can be generated by fungi as a carbon storage but also due to a low water activity or increased osmotic potential of the substrate (Tekelo *et al*., 2010; Kobayashi *et al*., 2015), conditions that might have been generated during the 8 weeks of incubation. Polyols, such as glycerol, fed up to 10% in the cattle diet, has no negative effect on intake and ruminal digestibility (Khalili *et al*., 1997; Schröder and Südekum, 1999), but can have a positive effect on milk production (Bodarski *et al*., 2005).

3.3.4 Mycotoxin content

Although it is unlikely that the basidiomycetes used here produce known mycotoxins, samples of untreated WS and 8 weeks fungal treated WS were analysed for the presence of known mycotoxins (Table 3.3). Except for Enniatin B in untreated WS, none of the other mycotoxins could be detected in untreated and treated WS. The absence of Enniatin B after 8 weeks might indicate that mycotoxins can also be degraded by *C. subvermispora* as has been reported for other white-rot fungi (Wang *et al*., 2011; Yehia, 2014). The analysis shows that mycotoxins are not an issue in using fungal treated WS as an animal feedstuff.

Table 3.3. Mycotoxins (mg kg^{-1} dry sample) analysed in wheat straw before (week 0) and after (week 8) *Ceriporiopsis subvermispora* and *Lentinula edodes* treatment.

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

* Mycotoxin content in another sample < 0.05.

3.4 Conclusions

Treatment of wheat straw with *C. subvermispora* and *L. edodes* results in a significant loss in dry matter and degradation of hemicellulose and lignin. GC-MS analysis showed changes in a number of organic acids and soluble sugars during the fungal treatment and there were clear differences between both fungi. Especially clear was the accumulation of polyols that reflects partly the amount of biomass formed, i.e. high amount in *L. edodes* and lower amounts in *C. subvermispora* treated WS. The largest changes of compound profiles by both fungi occurred within 4 weeks of incubation, where pH also showed the largest change. Four ceriporic acids (A, B, C and G) were observed and accumulated only during *C. subvermispora* treatment. No known mycotoxin were detected on *C. subvermispora* and *L. edodes* treated WS, indicating that those two fungal treated wheat straws are safe from the perspective of mycotoxin. The finding provides important information for further feeding activity in practice since those compounds might have either positive or negative effects on rumen microbes and palatability.

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Table S3.1. Specifications for mycotoxin identification.

Mycotoxin	Retention time	Precursor ion	Product ions	Adduct	Reporting limit	Recovery	RSD_{WLR} Recovery	Systemic deviation	Expended measurement uncertainty (U_f)	Origin
	(min)	(m/z)	(m/z)		(mg/kg)	(%)	(%)	(%)	(%)	
15-Acetyl- deoxynivalenol	3.3	355.9	321.1/137.1	[M+NH4]+	0.2	90	9.0	10	17	Sigma-aldrich
3-Acetyl- deoxynivalenol	3.4	397.1	337.0/59.0	[M+Ac]-	0.08	82	17	18	34	Sigma-aldrich
Aflatoxin B1	5.0	313.0	285.2/128.1	[M+H]+	0.0025	95	16	4.8	31	Sigma-aldrich (mix)
Aflatoxin B2	4.8	315.1	287.2/259.2	[M+H]+	0.0025	92	8.2	8.1	16	
Aflatoxin G1	4.4	329.0	243.2/200.0	[M+H]+	0.0025	94	9.6	6.2	19	
Aflatoxin G2	4.2	331.1	313.2/245.2	[M+H]+	0.005	99	13	1.3	26	
Agroclavine	3.2	239.1	183.2/208.2	[M+H]+	0.0025	81	15	19	29	Coring System DiagnostiX
Alternariol	6.0	257.0	212.9/214.9	[M-H]-	0.02	92	10	8	20	Coring System DiagnostiX
Alternariol-methyl ether	7.2	271.1	256.0/227.0	[M-H]-	0.005	88	9.9	12	20	Coring System DiagnostiX
Beauvericin	8.0	784.4	262.3/244.2	[M+H]+	0.025	86	22	14	45	Sigma-aldrich
Citrinin	4.4	281.2	249.2/205.2	[M-H]-	0.05	121	30	-21	60	Sigma-aldrich
Deoxynivalenol	2.7	297.0	249.0/231.0	[M+H]+	0.2	87	11	13	21	Sigma-aldrich
Diacetoxyscirpenol	4.3	384.2	307.2/105.1	[M+NH4]+	0.25	89	12	11	25	Coring System DiagnostiX
Deoxynivalenol-3-glucoside	2.7	517.2	457.1/247.0	[M+Ac]-	0.0125	73	12	27	25	Coring System DiagnostiX
Enniatin A	8.2	699.4	228.0/210.1	[M+NH4]+	0.05	92	16	8.4	33	BioAustralis
Enniatin A1	8.0	685.4	210.1/214.1	[M+NH4]+	0.05	96	13	3.6	26	BioAustralis

Enniatin B	7.7	657.5	214.1/196.3	[M+NH4]+	0.05	100	13	-0.3	27	BioAustralis
Enniatin B1	7.9	671.4	210.0/196.0	[M+NH4]+	0.05	96	14	4.1	29	BioAustralis
Fumonisin B1	5.4	722.5	334.4/352.3	[M+H]+	0.1	101	13	-1.0	27	Sigma-aldrich
Fumonisin B2	6.2	706.4	336.3/318.5	[M+H]+	0.04	101	13	-1.3	26	Sigma-aldrich
Fumonisin B3	5.9	706.4	336.3/318.5	[M+H]+	0.02	99	16	1.1	32	Coring System DiagnostiX
HT-2 toxin	5.1	441.9	215.1/263.1	[M+NH4]+	0.02	97	17	3.0	35	Coring System DiagnostiX
Moniliformin	0.9	96.6	41.2	[M-H]-	0.125	125	23	-25	47	Sigma-aldrich
Mycophenolic acid	5.7	338.1	303.2/207.0	[M+NH4]+	0.05	96	13	4.4	27	Coring System DiagnostiX
Nitropropionic acid	1.0	118.0	46.0	[M-H]-	0.05	87	19	13	37	Sigma-aldrich
Nivalenol	2.5	371.1	281.0/311.0	[M+Ac]-	0.1	73	13	27	26	Coring System DiagnostiX
Ochratoxin A	6.4	404.0	239.0/102.0	[M+H]+	0.002	90	17	10	34	Sigma-aldrich
Penicillic acid	3.0	168.9	110.0/92.9	[M+H]+	0.02	80	15	20	29	Coring System DiagnostiX
Roquefortine C	4.9	390.2	193.1/322.2	[M+H]+	0.0025	81	11	19	23	Coring System DiagnostiX
Sterigmatocystin	7.0	325.1	281.1/310.2	[M-H]-	0.001	97	9.5	2.8	19	Coring System DiagnostiX
T-2 Toxin	5.7	484.3	215.2/185.1	[M+NH4]+	0.02	95	14	5.0	27	Coring System DiagnostiX
Zearalenone	6.5	317.1	131.1/175.0	[M-H]-	0.05	94	14	6.0	28	Sigma-aldrich
α-Zearalenol	6.0	319.2	160.0/130.0	[M-H]-	0.01	100	12	-0.5	24	Sigma-aldrich
ß-Zearalenol	6.6	319.3	160.0/130.0	[M-H]-	0.01	88	12	12	25	Sigma-aldrich

Calibration curves were linear with correlation factors of 0.994 or higher. The reporting limit (or limit of quantification) was equal to the lowest validated mycotoxin concentration, and varied between mycotoxins. Average recoveries were in the range of 80–120%, with the exception of Deoxynivalenol-3-glucoside (73%), nivalenol (73%), citrinin (121%), and moniliformin (125%). Furthermore citrinin showed a relative within lab reproducibility (RSD_{WLR}) of 30% resulting in an overall relative measurement uncertainty U_r of 60%, which is outside of the requested range of 50%. As none of the samples were positive for citrinin this was regarded as of minor influence and the results were accepted.

Chapter 4

The influence of storage temperature and time on feed quality of fungal treated wheat straw

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Abstract

Degradation of lignocellulose by selective white-rot fungi can significantly improve the nutritional value of high lignocellulose containing biomass. To determine if such treated biomass is stable in time, wheat straw (WS) treated for 7 weeks with *Ceriporiopsis subvermispora* and *Lentinula edodes* was stored anaerobically up to 10 weeks at different temperatures (24.7-52.4 °C). The substrates were subsequently analysed for changes in pH, titratable acidity, fibre composition, *in vitro* gas production, colour and organic compounds using GC-MS and LC-MS. The titratable acidity of fungal treated WS increased with increasing storage temperatures and time, indicating acidification of straw. A small but significant decrease in hemicellulose and an increase in acid detergent lignin was observed at 52.4 °C. No effect of the storage was observed on the degradability of fungal treated WS in rumen fluid. A deeper colour was observed for substrate at higher temperatures, and a clear accumulation of organic acids and sugars during storage. The results show that, although some chemical changes occur, anaerobic storage of fungal treated WS at different temperatures does not change its nutritional value.

Key words

Ceriporiopsis subvermispora; *Lentinula edodes*; anaerobic storage; temperature; time.

4.1 Introduction

The aerobic treatment of lignocellulosic biomass, such as cereal straws, with white-rot fungi (e.g. *Ceriporiopsis subvermispora* and *Lentinula edodes*) has great potential to increase its feed value for ruminants, as well as the utilization in the production of chemicals and biofuels (Van Kuijk *et al*., 2015a). White-rot fungi degrade the lignin in the biomass and, thereby, increase the accessibility of the remaining polysaccharides for rumen microbiota. Especially selective white-rot fungi, such as *C. subvermispora* and *L. edodes*, show a great delignification ability with a low simultaneous utilization of cellulose (Tuyen *et al*., 2012). The fungi are able to cleave the lignocellulose linkages and degrade lignin in the biomass by secreting oxidative enzymes and diverse low molecular mass radicals (Hammel *et al*., 2002; Ohashi *et al*., 2011; Pollegioni *et al*., 2015). During colonization, fungal hyphae mainly accumulate in the lumen of plant cells where enzymes are secreted to degrade the cell wall. The plant cell walls, are too dense to allow the penetration by enzymes. This hurdle is overcome by the production of small radicals that can diffuse into the cell wall and break down lignin (Blanchette *et al*., 1997).

White-rot fungi decompose lignin as well as carbohydrates to utilise the carbohydrates as a carbon and energy source to meet the requirements for growth and activity, hence a longer fungal incubation shows higher losses of dry matter and carbohydrates (Van Kuijk *et al*., 2015b; Nayan *et al*., 2017). As such, the timing of terminating fungal activity is essential to optimise the nutritional value of the biomass. Several studies have shown that substrates acidify during the aerobic culture with *C. subvermispora* and *L. edodes* (Mäkelä *et al*., 2002; Kwak *et al*., 2016; Mao *et al*., 2018). Wheat straw treated with *C. subvermispora* and *L. edodes* can thus be stored under anaerobic conditions at 20 °C up to 64 days, without using additives (Mao *et al*., 2018). Although fungi show slow or no further decomposition of lignin and cell wall components during anaerobic storage, some fermentation activity still occurs for, hitherto, unknown reasons (Yang *et al*., 2001; Mao *et al*., 2018). In order to be applicable as an commonly used animal feedstuff, fungal threated biomass must be able to be stored for extended periods of time and retain its nutritional quality under variable climatic conditions.

The aim of the present research was to investigate the effect of storage of fungal treated wheat straw (WS) in the temperature range of 24.7-52.4 °C during 10 weeks on its nutritional quality. The high temperatures were used as an accelerated shelf-life test (Kebede *et al*., 2015). Changes were measured in pH, fibre composition, digestibility by

estimating *in vitro* gas production (IVGP) and metabolite level using gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS).

4.2 Materials and methods

4.2.1 Fungal strains and spawn preparation

The fungi *C. subvermispora* (CBS 347.63; USA) and *L. edodes* (CCBAS389; Czech Republic) were recovered from the liquid nitrogen collection of Plant Breeding (Wageningen University & Research, The Netherlands), and mycelium was grown on malt agar plates containing 10 g l^{-1} malt extract (Oxoid Ltd, Hampshire, UK) and 17.5 g l^{-1} micro agar (Duchefa Biochemie B.V, Haarlem, The Netherlands) at 25 °C for one to two weeks (depending on the fungus) until the agar plate was fully covered. Two agar pieces (~ 1.5 cm^2) were then transferred to plastic boxes (OS60 + OD60; Combiness, Nevele, Belgium) containing approximately 75 g of sterilized sorghum grain and incubated in a climate controlled room at 25 °C. The fully colonized grains were stored at 4 °C for further use.

4.2.2 Aerobic culture of fungi on wheat straw

Organic wheat (*Triticum aestivum* L.) straw was obtained locally and mechanically (Pierret Industries, Corbion, Belgium) chopped into pieces of 0.5-1.5 cm. The WS was filled in monofilament net bags (50 × 80 cm, pore size: 0.15 × 0.15 cm) and immerged in tap water for three days and then drained for 5 h. The fungal inoculation and incubation with *C. subvermispora* and *L. edodes* was carried out in 4 l plastic containers (TP4000 + TPD4000; Combiness, Nevele, Belgium) containing a filter in the lid allowing gas exchange. Before fungal inoculation, all containers filled with mixed wet WS (dry weight of WS in each container was approximate 160 g) were autoclaved at 121 °C for 1 h and then left overnight in a flow cabinet. The substrate comprised of untreated autoclaved WS (AWS), *C. subvermispora* treated AWS (CS-AWS) and *L. edodes* treated AWS (LE-AWS). Sixteen g of spawn of each fungus was aseptically inoculated on the AWS, gently mixed by hand in a flow cabinet and then incubated at 25 °C for 7 weeks in a climate controlled room.

4.2.3 Anaerobic storage

After 7 weeks of aerobic incubation, each box of AWS, CS-AWS and LE-AWS was mixed and transferred into 0.5 l glass jars, sealed by a locking lid with rubber seal. The substrates in the glass jars were manually compacted as much as possible to press out air. The jars were stored in a climate controlled room with an average temperature of 24.7 °C, and in ovens with an average temperature of 35.0, 45.9 and 52.4 °C. Data loggers (LIBERO CB; ELPRO, Buchs SG, Switzerland) were used to monitor the temperature at 1 h intervals during the storage period. Subsequently, three glass jars for each temperature were opened after 2, 4, 6, 8 and 10 weeks of storage. In the case of week 0, substrate was collected directly after the aerobic incubations, without filling glass jars. Approximately 20% of the content of the glass jars was stored at -20 °C for further analysis of pH and assessing titratable acidity. The remainder of the sample was freeze dried prior to the analysis of the chemical composition, the IVGP and colour measurements.

4.2.4 Analytical methods

4.2.4.1 Chemical composition

Freeze dried samples were ground in a hammer mill over a 1 mm sieve (100 AN; Peppink, Olst, The Netherlands). Residual dry matter (DM) content of the freeze dried material was determined after drying in an oven at 103 °C for 4 h (ISO 6496, 1999) and subsequently ash content was determined after incinerating in a muffle furnace at 550 °C for 3 h (ISO 5984, 2002). The nitrogen (N) content was analysed by the Kjeldahl method (ISO 5983, 2005). Acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) were analysed according to the methods described by Van Soest *et al*. (1991), using an Ankom fiber analyzer (A2000I; ANKOM Technology, Macedon, NY, USA). The cellulose content was calculated as the difference between ADF and ADL, and hemicellulose as the difference between NDF and ADF.

Water extracts were made by mixing 30 g of fresh sample with 270 ml of demineralised water and homogenised using a stomacher (400 Circulator; Seward, Worthing, UK) at 230 rpm for 5 min. The initial pH of the stomacher extracts was determined and subsequently the extract was titrated with 0.1 mol l^{-1} NaOH (Titrisol sodium hydroxide solution; Merck, Darmstadt, Germany) to pH 7 using a titrando machine (Metrohm, Herisau, Swizerland). Titratable acidity was calculated as mmol NaOH per kg DM substrate required to achieve pH 7.

4.2.4.2 *In vitro* gas production

In vitro gas production was performed according to Cone *et al*. (1996). In brief, rumen fluid was collected from three lactating, rumen fistulated cows fed *ad libitum* maize silage and grass silage. The strained rumen fluid was filtered through two layers of cheese cloth and mixed with a mineral buffer solution. All procedures were conducted under continuous flushing with CO_2. Approximately 0.5 g of sample was incubated with 60 ml of buffered rumen fluid for 72 h and gas production was recorded automatically. Gas production was corrected for blank gas production (i.e. gas production in buffered rumen fluid without sample).

4.2.4.3 Colour measurement

The colour of the freeze dried samples was measured by a colorimeter (CR-200; Minolta, Japan). The parameter 'L' indicates the lightness of the sample from value 0 (black) to 100 (white). The parameter 'a' indicates a negative value with green and a positive value with red. 'b' indicates a negative value with blue and a positive value with yellow. The colour parameters 'L', 'a' and 'b' were used to show the colour change in brightness, redness and yellowness. The instrument was calibrated with a white calibration plate with parameter L = + 97.79; a = - 0.44; b = + 2.04.

Overall change in colour (ΔE1) of samples was calculated from the parameters 'L', 'a' and 'b' according to the following equation:

(1) $\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2}$

where L_0, a_0 and b_0 represent the control values, being AWS and AWS incubated with *C. subvermispora* and *L. edodes* for 7 weeks. 'L', 'a' and 'b' represent the values of the corresponding stored samples.

4.2.4.4 Compounds analysed in fungal treated wheat straw

The relative content of various components was determined by GC-MS. The compounds from CS-AWS and LE-AWS were extracted according to the methods described by Lisec *et al*. (2006). Modifications were performed aimed for a better extraction of components from the freeze dried materials. In brief, approximately 50 mg (48-52 mg) ground sample was weighed in a 2 ml Eppendorf tube, with 1.4 ml of 80% pre-cooled methanol and 60 µl of ribitol (0.2 mg ml^{-1}, internal quantitative standard) was added. The extracts were vortexed for 10 s, and subsequently incubated in a thermomixer (Vortemp 56; Labnet

International Inc., Edison, USA) at 950 rpm at 70 °C for 10 min, followed by centrifugation for 10 min at top speed (21000 × g) in a Eppendorf centrifuge. Afterwards, 500 µl of supernatant was transferred in a new Eppendorf tube and mixed with 375 µl of chloroform and 750 µl of H_2O for 10 s, and the phases were separated by centrifugation at top speed (21000 × g) in a Eppendorf centrifuge. Subsequently 100 µl of the methanol-water phase was dried in glass vials by vacuum centrifugation.

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

Ceriporic acids A, B, C and G were measured by LC-MS. The extraction of fungal treated WS for the LC-MS analysis was performed using a protocol as described by De Vos *et al*. (2007). In short, approximate 100 mg (97-103 mg) of ground sample was weighed in the 2 ml Eppendorf tube, and extracted with 1.5 ml of 75% methanol and 0.1% of formic acid. The extract was vortexed for 10 s and sonicated for 10 min. The mixture was then centrifuged for 10 min in an Eppendorf centrifuge (16000 × g), an aliquot of supernatant (180 µl) was transferred to a glass vial with insert for LC-MS analysis. Chromatographic separation, mass detection methods and the equipment are described by Mokochinski *et al*. (2018). The mass spectra of ceriporic acid A, B and C were identified according to Amirta *et al*. (2003) and Van Kuijk *et al*. (2017) while mass spectra of ceriporic acid G was according to Nishimura *et al*. (2012a).

4.2.5 The metabolite data processing and statistical analysis

The data processing and statistical analysis of the GC-MS and LC-MS analysis was performed according to steps described by Mokochinski *et al*. (2018). The principal component analysis (PCA) was performed using the software Simca (version 14).

All measured parameters were evaluated for their main effects of storage temperature and storage time and the interactions of the two factors were analysed by the GLM procedure in SAS 9.3 version. Changes in pH, titratable acidity, IVGP and colour were measured

after 0, 2, 4, 6, 8 and 10 weeks of storage. Moreover, the chemical composition was determined in samples stored for 0, 2 and 10 weeks. Significance was declared at $P < 0.05$.

4.3 Results and discussion

4.3.1 Change in pH and titratable acidity during anaerobic storage

Changes in pH and titratable acidity (mmol NaOH per kg DM required to reach pH 7) of AWS, CS-AWS (*C. subvermispora* treated AWS) or LE-AWS (*L. edodes* treated AWS) stored anaerobically at 24.7, 35.0, 45.9 and 52.4 °C are illustrated in Fig. 4.1 panel A-F. During the storage of AWS at different temperatures for up to 10 weeks, only minor changes in pH were observed (Fig. 4.1A). A clearly increasing and higher titratable acidity of AWS was seen when stored at 52.4 °C (Fig. 4.1B) likely caused by some enzymatic but more likely chemical reactions in the organic matter. The initial pH of the 7 weeks fungal treated AWS at the start of the anaerobic incubation was 3.47 and 4.18 for *C. subvermispora* or *L. edodes*, respectively (Fig. 4.1C and E). A low pH seems to be a vital factor for fungi for a quicker growth and more efficient lignin degradation (Magnuson and Lasure, 2004). These results are in line with those of Mäkelä *et al*. (2002) who found in other white-rot fungi that the decrease in pH was caused by secretion of acids. Regardless of the temperature, a significant reduction in pH value during the first 2 weeks of storage of CS-AWS (3.17-3.30) and LE-AWS (3.74-3.92) was observed in comparison with the start of the anaerobic incubation, indicating that the acidity of the fungal treated AWS changed after storage under anaerobic conditions. This might indicate an accelerated acid formation either by the still active excreted fungal enzymes or chemical reactions. The former might be explained by the continuous metabolic activity of the fungi under decreasing oxygen concentrations. Growth of basidiomycete fungi can go on for a short time under low oxygen conditions and change the chemistry of decomposition of lignocellulose (Nsolomo *et al*., 2000; Pavarina and Durrant, 2002), which might also explain to some extent the decrease in pH during the first 2 weeks of anaerobic incubation. It is, however, also possible that chemical reactions increased the amount of acids in fungal treated AWS. Ambye-Jensen *et al*. (2013) have shown that in ensiled WS acidified by bacteria, the amount of acids increased with storage at increasing temperature. Although they used higher temperatures, it is likely that with storage of WS acidified by fungi, similar reactions take place. During storage, temperature ($P < 0.001$)

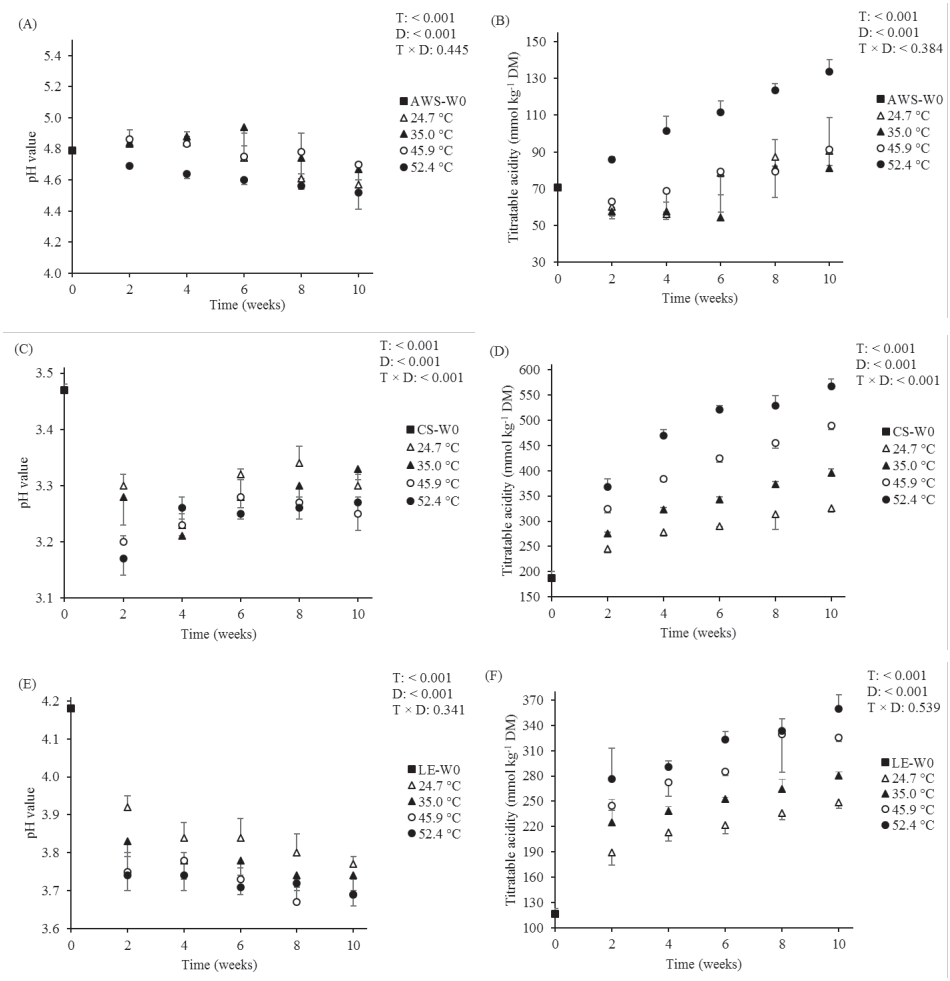

Fig. 4.1. Changes in pH and titratable acidity of autoclaved wheat straw (AWS) (A and B), *Ceriporiopsis subvermispora* (CS) treated AWS (C and D) and *Lentinula edodes* (LE) treated AWS (E and F) stored anaerobically under different temperatures (T: 24.7, 35.0, 45.9 and 52.4 °C) and duration (D: 2, 4, 6, 8 and 10 weeks). W0 represent the start of anaerobic storage (AWS aerobically incubated without or with CS and LE at 25 °C for 7 weeks).

and duration ($P < 0.001$) significantly affected the pH of CS-AWS and LE-AWS. No interaction was found between temperature and time for LE-AWS ($P = 0.341$). No clear decline in pH was observed for CS-AWS during storage, while a clear (but small) trend for a decrease in pH from week 0 to 10 for LE-AWS was observed.

Apart from pH, the type and amount of acids produced by fungi should also be considered. *C. subvermispora* treated AWS (187.5 mmol kg^{-1} DM) showed a higher initial titratable

acidity than LE-AWS (116.6 mmol kg^{-1} DM) at week 0 (Fig. 4.1D and F), indicating a higher amount or different types of acids were produced by *C. subvermispora*. As for the pH data, a sharp increase in titratable acidity during the first 2 weeks of storage of CS-AWS (245.1-368.1 mmol kg^{-1} DM) and LE-AWS (189.7-276.3 mmol kg^{-1} DM) was observed in comparison with week 0. There was a tendency of an increasing difference in titratable acidity between temperatures towards 10 weeks for CS-AWS, whereas for LE-AWS this remained the same. Part of the increase in titratable acidity with temperature and time was also seen in AWS but the major changes were due to the fungal treatment. No interaction was seen between storage temperature and duration for the LE-AWS ($P = 0.539$).

4.3.2 Change in chemical composition of fungal treated wheat straw

The stored untreated and fungal treated AWS stored for 0, 2 and 10 weeks at all temperatures were also analyzed for chemical composition (Table 4.1 and 4.2). No interaction between storage temperature and duration was found with ash, nitrogen, cellulose and hemicellulose content in AWS. For ADL, three of the four temperatures showed an increase (Table 4.1). No significant change was found for the ash content in CS-AWS and LE-AWS during the storage at different temperatures, indicating that the total organic matter remained stable during anaerobic storage (Table 4.2). The content of cellulose was significantly affected by temperature (CS: $P = 0.015$; LE: $P < 0.001$), while hemicellulose and ADL in both CS-AWS and LE-AWS were significantly affected by both storage temperature and time ($P < 0.05$). A reduction in hemicellulose content at all temperatures was observed from 2 to 10 weeks of storage, which was significant ($P < 0.05$) at 52.7 °C in CS-AWS, and at 45.9 °C and 52.4 °C in LE-AWS. The reverse trend was observed for the ADL content, except for a small increase at 24.7 °C in CS-AWS. Although fungal growth and excretion of enzymes will decrease sometime after the onset of anaerobic storage, a continuing activity of hydrolytic enzymes might lead to some degradation of hemicellulose. No significant changes of ADL was expected since oxygen is required for degradation of lignin. It has been reported that the disappearance of carbohydrates, mainly hemicellulose, also can be the result from the acidic environment during ensiling (Morrison, 1979; Singh *et al*., 1996). Hence, acidic conditions generated by fungi might also be a reason for hemicellulose hydrolysis or solubilisation during the

Table 4.1. Chemical composition of autoclaved wheat straw stored anaerobically at different temperatures and duration.

Temperature (°C)	Duration (Week)	Ash	Nitrogen	Cellulose	Hemicellulose	ADL
		(g kg^{-1} dry matter)				
Control	0	38.6	2.51	468.0	288.8	68.2
24.7	2	38.6	2.49	474.2	275.9	66.6^{bc}
	10	38.9	2.43	473.6	272.5	68.8^{abc}
35.0	2	39.0	2.15	470.8	280.6	67.7^{bc}
	10	39.3	1.86	468.1	276.9	65.7^{c}
45.9	2	37.6	1.91	475.3	273.0	68.8^{abc}
	10	37.6	2.00	470.7	273.8	70.9^{ab}
52.4	2	37.1	1.96	474.1	280.8	68.9^{abc}
	10	38.5	1.99	461.6	268.0	73.2^{a}
RMSE		1.22	0.123	5.41	6.37	1.70
P value						
Temperature		0.133	< 0.001	0.212	0.483	0.002
Duration		0.339	0.305	0.034	0.084	0.028
Temperature × Duration		0.764	0.108	0.285	0.335	0.037

ADL, acid detergent lignin; RMSE, root mean square error.

$^{a-c}$ Values with different superscripts within a column are significantly ($P < 0.05$) different.

anaerobic storage, especially at an elevated storage temperature and for a prolonged period. Yang *et al*. (2001) conserved *Penicilium decumbens* treated corn straw (with wheat bran) anaerobically at 25, 30, 35 and 40 °C with addition of lactic acid bacteria and molasses for pH reduction. These authors showed that reducing sugars are increased with a longer storage period and at higher temperatures after an initial decrease at the beginning of the storage period.

4.3.3 Changes of IVGP caused by storage

The IVGP is an effective indicator for the degradability of organic matter in buffered rumen fluid (Cone *et al*., 1996). Aerobic incubation for 7 weeks with *C. subvermispora* and *L. edodes* increased the IVGP from 246.2 to 288.4 ml g^{-1} OM and 221.5 to 244.7 ml g^{-1} OM, respectively. The IVGP of untreated and fungal treated AWS during 10 weeks of anaerobic storage showed no significant changes (Fig. 4.2). Although a significant decrease in hemicellulose content occurred in CS-AWS and LE-AWS ($P < 0.05$) (Table 4.2) stored anaerobically at 52.4 °C, this did not affect the IVGP. The method used to

Table 4.2. Chemical composition of *Ceriporiopsis subvermispora* and *Lentinula edodes* treated autoclaved wheat straw stored anaerobically at different temperatures and duration.

Temperature (°C)	Duration (Week)	*C. subvermispora* (g kg^{-1} dry matter)					*L. edodes* (g kg^{-1} dry matter)				
		Ash	Nitrogen	Cellulose	Hemicellulose	ADL	Ash	Nitrogen	Cellulose	Hemicellulose	ADL
Control	0	43.3	3.58	497.6	141.1	26.9	42.3	3.72	507.5	164.9	45.3
24.7	2	42.4	3.50	495.7	135.7^{a}	27.7^{bc}	41.9	3.89^{a}	503.9^{de}	158.2^{ab}	42.7
	10	42.8	3.71	494.2	124.2^{a}	26.7^{c}	39.9	2.51^{b}	504.0^{de}	158.1^{ab}	47.1
35.0	2	43.0	3.56	498.1	135.6^{a}	28.5^{bc}	41.0	2.48^{b}	505.1^{cde}	158.8^{ab}	43.2
	10	43.7	3.66	495.5	133.3^{a}	29.7^{bc}	41.5	2.55^{b}	498.6^{e}	158.4^{ab}	43.5
45.9	2	42.5	3.66	502.3	134.5^{a}	26.4^{c}	41.2	2.47^{b}	508.2^{bcd}	167.6^{a}	46.1
	10	42.6	3.68	501.0	115.3^{a}	31.9^{b}	41.4	2.44^{b}	512.1^{abc}	144.1^{bc}	47.8
52.4	2	42.9	3.64	503.2	128.0^{a}	30.2^{bc}	41.1	3.33^{a}	513.7^{ab}	155.6^{ab}	48.3
	10	43.7	3.56	513.4	80.7^{b}	40.4^{a}	41.2	3.85^{a}	516.5^{a}	135.5^{c}	55.4
RMSE		0.896	0.171	6.69	7.70	1.65	1.25	0.223	2.87	6.36	3.21
P value											
Temperature		0.296	0.885	0.015	< 0.001	< 0.001	0.939	< 0.001	< 0.001	0.008	0.002
Duration		0.152	0.384	0.675	< 0.001	< 0.001	0.555	0.037	0.950	< 0.001	0.020
Temperature × Duration		0.880	0.521	0.332	< 0.001	< 0.001	0.329	< 0.001	0.027	0.007	0.300

ADL, acid detergent lignin; RMSE, root mean square error.

[a-e] Values with different superscripts within a column are significantly ($P < 0.05$) different.

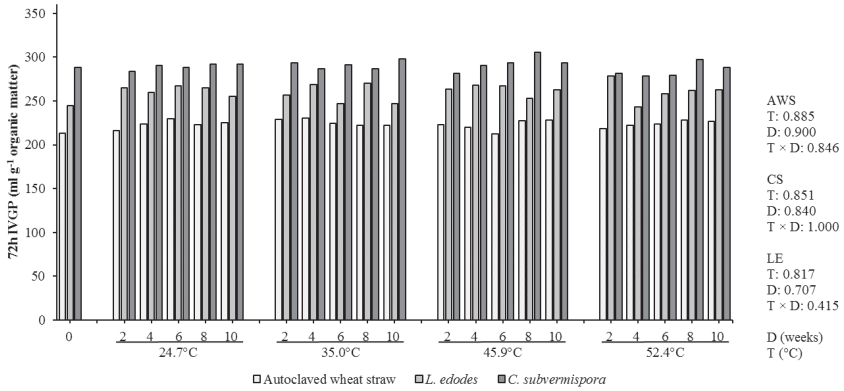

Fig. 4.2. Cumulative (72h) *in vitro* gas production (IVGP) of autoclaved wheat straw (AWS), *Ceriporiopsis subvermispora* (CS) treated AWS and *Lentinula edodes* (LE) treated AWS stored anaerobically under different temperatures (T: 24.7, 35.0, 45.9 and 52.4 °C) and duration (D: 2, 4, 6, 8 and 10 weeks). Week 0 represent the start of anaerobic storage (AWS aerobically incubated without or with CS and LE at 25 °C for 7 weeks).

assess the amount of carbohydrate fibres such as cellulose and hemicellulose is based on gravimetric measurements of residues after extraction (Van Soest *et al*., 1991). This apparent discrepancy between the fibre content (Table 4.2) and IVGP results (Fig. 4.2) can be explained by an increase in solubility and conversion into free sugars of the hemicellulose. The latter will not affect IVGP results but does affect the measurement of hemicellulose.

4.3.4 Colour change during anaerobic storage at different temperatures

Both *C. subvermispora* and *L. edodes* caused a significant change in colour after 7 weeks of incubations from L: 70.6, a: 2.03, b: 19.5 in AWS to L: 75.1, a: 3.54, b: 25.5 after *C. subvermispora* treatment and to L: 74.7, a: 3.04, b: 23.0 after *L. edodes* treatment. This shows that the colour of WS became lighter, redder and yellower. This is in line with Arora *et al*. (2011), who indicated that redness and yellowness are higher in *C. subvermispora* treated WS than control WS. However, they reported a slightly lower L-value in *C. subvermispora* treated WS, which is opposite to the results in the current study. Fungal degradation of biomass results in a variety of changes in physical, biological and

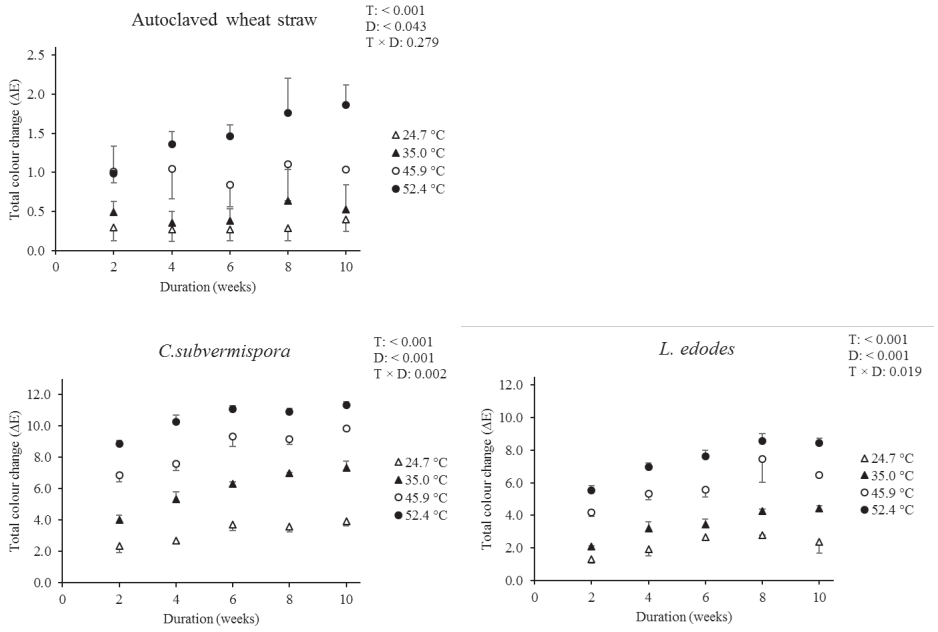

Fig. 4.3. Changes in colour of autoclaved wheat straw (AWS), *Ceriporiopsis subvermispora* treated and *Lentinula edodes* treated AWS stored anaerobically under different temperatures (T: 24.7, 35.0, 45.9 and 52.4 °C) and duration (D: 2, 4, 6, 8 and 10 weeks). ΔE is the colour difference between stored fungal treated AWS and control, which represent the start of anaerobic storage (AWS aerobically incubated without or with fungus at 25 °C for 7 weeks).

chemical properties (Blanchette *et al*., 1997; Tuyen *et al*., 2012; Van Kuijk *et al*., 2015b). White-rot fungi generally have a bleaching effect on lignocellulose since lignin and hemicellulose are removed in the earlier stage of colonization, leading to an enrichment in "whitish" cellulose (Hatakka and Hammel, 2011; Martínez *et al*., 2011).

Overall change in colour of AWS and fungal treated AWS stored at different temperatures and for different periods is shown in Fig. 4.3. ΔE of untreated and treated WS was significantly affected by temperature ($P < 0.001$) and time ($P < 0.05$). A clear increase in ΔE of AWS was seen when stored at 52.4 °C (Fig. 4.3). A rapid increase in ΔE value was observed especially in the first 2 weeks and increasing more gradually up to 6 weeks of storage of CS-AWS and 8 weeks of storage of LE-AWS at all temperatures to remain more or less stable thereafter (Fig. 4.3). The ΔE is mainly influenced by a decrease in the L-value. The two common reactions known to increase brownness are the Maillard reaction and caramelisation. It is possible that browning by the Maillard reactions occurs

as a result of the accumulation of reducing sugars in the fungal treated substrates (Yang *et al*., 2001), which are able to react with amino groups. Caramelisation can also contribute to the colour change during the first few days when there is still oxygen present. The changes in colour might also be caused in part by changes in polyphenols during storage. Since the major change in colour occurs at the beginning of the storage (between 0 and 2 weeks), it is possible that polymerization of mono-phenols occurs by laccase activity using the remaining oxygen present. Polyphenols are dark brown, dark orange, and dark wine red (Sun *et al*., 2013) and the increase in the ADL content with temperature and storage would indicate that polymerization of mono-phenols may have occurred.

4.3.5 The compounds from stored fungal treated wheat straw

The compounds in CS-AWS and LE-AWS before and after storage at 24.7 and 52.4 °C for 0, 2 and 10 weeks were analysed by GC-MS and LC-MS. The data represent relative abundancy and not absolute amounts since not for all components standards were included. To provide an overview of the change of all components (identified and unknown) from GC-MS, a principle component analysis (PCA) was conducted. The close grouping of biological replicates in the PCA plots indicates a high reproducibility of the analytic methods used. The first two principle components explain 53.2% of the variance for the GC-MS data (Fig. 4.4). The plot indicates clear effects of storage temperature and storage time on the released compounds profile, and *C. subvermispora* and *L. edodes* could be clearly distinguished as separate groups. For GC-MS, the largest distance for both principle components at 24.7 °C and 52.4 °C was between week 0 and 2 indicating that the largest changes occurred at the beginning of anaerobic incubation. The latter correlates with the changes in pH, titratable acidity and colour, which also show the major changes within the first 2 weeks of storage. At 24.7 °C, the largest changes are explained by principle component one and when stored at 52.4 °C additional changes are explained by principle component two.

The compounds observed and annotated using GC-MS were broadly classified into three groups, organic acids, carbohydrates and others (Table 4.3). For simplicity, only data of 2 and 10 weeks at 24.7 and 52.4 °C were used. The levels of organic acids in CS-AWS and LE-AWS were different at the beginning of the storage (week 0), indicating that the

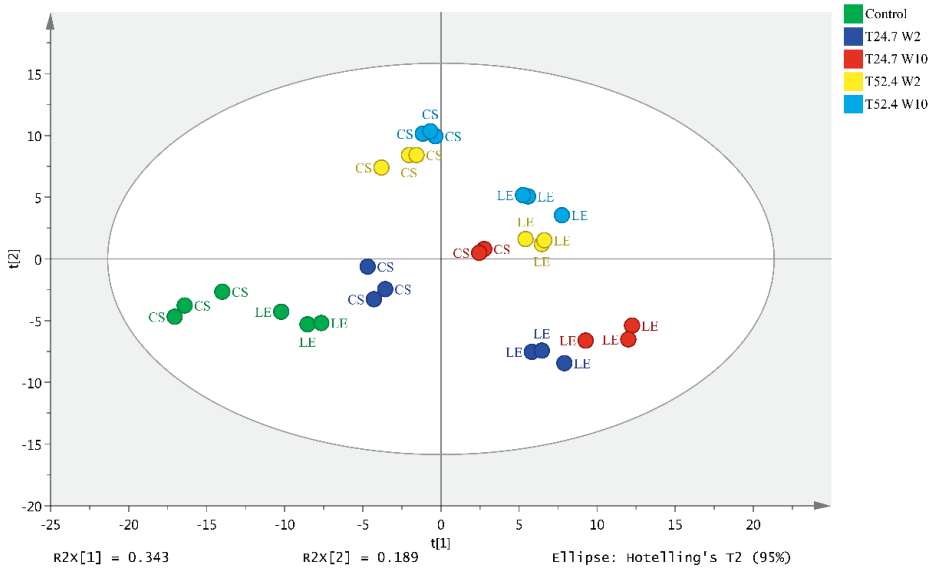

Fig. 4.4. The principal components analysis showing the major differences of *Ceriporiopsis subvermispora* (CS) and *Lentinula edodes* (LE) treated autoclaved wheat straw (AWS) stored anaerobically at 24.7 °C (T24.7) and 52.4 °C (T52.4) for 2 (W2) and 10 (W10) weeks, analysed via gas chromatography mass spectrometry (GC-MS). The control represent the start of anaerobic storage (AWS aerobically incubated with CS and LE at 25 °C for 7 weeks).

two fungi accumulated different organic acids during the aerobic incubation of 7 weeks (Chapter 3).

Among the organic acids analysed, the increase in glycolic and glyceric acids is notable. The increase is positively affected by time and temperature. It is unlikely that these compounds are only produced by enzymes excreted by the fungi and are likely produced by an unknown chemical reaction. The same accounts for the formation of 2,3,4-trihydroxybutyric acid, malic acid, tartaric acid and ribonic acid. The former three also accumulates in time especially at higher temperatures. Since 2,3,4-trihydroxybutyric acid, malic acid and ribonic acid accumulate at higher concentrations in LE-AWS, the differences in chemical composition of the treated substrate might have an effect on the formation of these compounds. Vanillic acid and protocatechuic acids are intermediates of lignin degradation (Mäkelä *et al*., 2015) and accumulate to some extent indicating that lignin degradation can continue after the onset of anaerobic incubation. The levels of

Table 4.3. Compounds released from *Ceriporiopsis subvermispora* and *Lentinula edodes* treated wheat straw stored anaerobically at 24.7 °C (T24.7) and 52.4 °C (T52.4) for 0 (W0), 2 (W2) and 10 (W10) weeks.

					C. subvermispora					*L. edodes*				
Compound	Centrotype	RIexp	Name	Annotation level*	W0	T24.7 W2	T24.7 W10	T52.4 W2	T52.4 W10	W0	T24.7 W2	T24.7 W10	T52.4 W2	T52.4 W10
Organic acid	303	1074	Lactic Acid, 2TMS	1	0	382	1320	2278	1760	55	45	207	114	466
	514	1088	Glycolic acid, 2TMS	1	1740	8096	17166	34542	55540	1478	7218	14669	34540	50374
	1076	1141	Oxalic acid, 2TMS	1	8806	8076	9784	7240	7057	7590	9015	8445	8922	7713
	2828	1301	Succinic acid, 2TMS	1	516	7329	7942	8516	11078	1078	5550	6066	7269	9746
	2965	1312	Glyceric acid, 3TMS	2	4867	12448	26531	40623	57660	8161	14171	29485	41924	65710
	3675	1384	Formic acid, 1TMS	2	2321	2236	2384	2149	2629	132	737	1299	1018	1734
	3756	1392	Malonic acid, 2TMS	2	81	244	728	321	682	125	1284	3178	2918	3308
	4535	1473	Malic acid, 3TMS	1	206	448	831	2148	2794	1577	4131	7250	20917	20668
	5045	1515	Pipecolic acid, 2TMS	2	191	573	1541	2111	3015	868	1159	2279	3470	5546
	5362	1543	2,3,4-Trihydroxybutyric acid, 4TMS	2	2194	6158	11334	9181	12280	1424	8775	18015	14914	19845
	6103	1623	Tartaric acid, 4TMS	1	2137	2919	3823	4628	6517	668	481	875	1642	2296
	8528	1749	Ribonic acid, 4TMS	1	3615	11802	24256	9118	15048	3223	15029	35210	26615	37517
	8758	1765	Vanillic acid, 2TMS	1	4921	5970	6659	10205	14831	3549	4247	6325	7926	9809
	9263	1816	Protocatechuic acid, 3TMS	1	314	340	683	1344	1809	130	93	179	526	974
	11840	1924	Galacturonic acid, 5TMS, methyloxime 1	1	12	0	0	0	0	2982	2464	2482	1912	1555
Carbohydrate	3340	1345	Dihydroxyacetone, 2TMS	2	1323	2036	3229	3271	3364	1485	2455	2102	2851	2293
	4761	1489	Threitol, 4TMS	1	423	5975	6810	5080	7617	921	3523	4572	4181	6138
	6170	1627	Arabinose, 4TMS, methyloxime	1	2452	3488	4147	5065	5893	2441	2144	3086	3396	5044

	6502	1636	Arabinose, 4TMS, methyloxime, metyloxime (isomer 2)	1	2632	21060	35224	35203	72447	8262	71697	166775	147619	200522
	7129	1647	Xylose, 4TMS, methyloxime (anti)	1	10486	181217	550238	801498	1856391	47640	455837	1055308	603618	1470409
	7529	1662	Xylose, 4TMS, methyloxime(syn)	1	426	1073	1648	2899	6255	1142	4533	9560	6145	10581
	7959	1704	Arabitol, 5TMS	1	107622	44076	69699	68527	59056	183294	211731	204031	195803	168479
	9832	1857	Fructose, 5TMS, methyloxime (anti)	1	1569	20069	50164	69412	61680	2105	12829	33919	106493	102331
	10004	1867	Fructose, 5TMS, methyloxime (syn)	1	871	14570	37003	53086	48990	1201	5610	18909	82844	80112
	10263	1877	Mannose, 5TMS, methyloxime (1Z)	1	665	14488	39414	7768	17461	2340	48802	102114	16858	23620
	10566	1883	d-Glucose, 5TMS, o-methyloxyme, (1E)	1	7989	541724	1195726	272283	292221	14657	1225119	1564505	616553	552862
	11099	1903	d-Glucose, 5TMS, o-methyloxyme, (1Z)	1	1625	112224	263684	54267	58290	3343	263592	502472	122071	116492
	11450	1914	Mannitol, 6TMS	1	48626	62949	48047	56719	57849	381193	649740	447671	523277	549200
	12090	1941	Myo-Inositol, 6TMS	1	4988	4071	2234	5628	5686	5837	5921	2893	5118	5169
	13205	2074	Myo-Inositol, 6TMS	2	1955	7101	9363	6265	7300	5795	11348	13183	9840	10553
	14498	2356	Xylobiose, 6TMS, methyloxime 1	1	3658	325162	722229	165269	249002	41679	182714	154089	16835	32379
	14812	2376	Xylobiose, 6TMS, methyloxime 2	1	908	93431	186725	47070	65997	12454	54766	43402	10110	10788
	16191	2545	Sucrose, 8TMS	1	11903	10151	6971	2501	5068	21030	21408	20050	5669	3927
	16763	2625	Maltose, 8TMS, methyloxime (isomer 1)	1	66	4426	5374	651	1608	70	86	308	992	1457
	16978	2631	Trehalose, 8TMS	1	5582	44	70	3639	4407	25502	197	934	22234	20517
	17820	2779	Uridine, 3TMS	1	245	174	364	9112	1398	1446	532	506	15999	9806
Others	2308	1257	Glycerol, 3TMS	1	15775	108825	108793	60130	70920	15287	42116	55027	39826	46789

* The annotation was levelled according to the rules described by Sumner *et al*. (2007).

those phenolic acids are higher at week 0 in CS-AWS than LE-AWS as expected since *C. subvermispora* degrades more lignin (Nayan *et al*., 2018). Oxalic acid, known to be produced by white-rot fungi (Galkin *et al*., 1998; Mäkelä *et al*., 2002; Aguiar *et al*., 2006; Kwak *et al*., 2016) was present in both treated substrates and remained fairly constant during the storage. Acids that are only produced by *C. subvermispora* are ceriporic acids (Enoki *et al*., 2002; Amirta *et al*., 2003; Nishimura *et al*., 2008; Nishimura *et al*., 2012a, 2012b). Using LC-MS, the relative amount of ceriporic acid A, B, C and G were determined. The amount of most of these acids increased slightly at lower temperature in time and decreased in time at high temperature (Fig. 4.5). No galacturonic acid was found in CS-AWS while detected in LE-AWS. Galacturonic acid is a major component of pectin (Mäkelä *et al*., 2015), the difference between *C. subvermispora* and *L. edodes* might be due to the continuing pectin degradation during the first weeks of anaerobic incubation by *L. edodes* either by its higher activity of pectin degrading enzymes or merely due to the higher amount of fungal biomass of *L. edodes* compared to *C. subvermispora*. All in all, the relative differences in the measured acids cannot explain well the differences seen in titratable acidity between CS-AWS and LE-AWS indicating that not all acids could be identified. Minor peaks of malic acid, tartaric and malonic acids were found in aqueous extracts from *C. subvermispora* treated *Pinus taeda* wood chips, while tartaric, citric, succinic and malonic acids were detected in liquid cultures of *C. subvermispora* with glucose as the single carbon source (Aguiar *et al*., 2006). Malic, succinic and tartaric acid were also reported in the fruit body of *L. edodes* (Chen *et al*., 2015).

Numerous sugars increased during storage, including monosaccharides and disaccharides (Table 4.3). In contrast with un-stored fungal treated WS, pentose (xylose, arabinose), and hexoses (mannose, glucose and fructose) accumulated in both fungal treated straw. The accumulation of these sugars might be partly caused by the continuous activity of plant cell wall degrading enzymes during storage and leakage of sugars accumulated in hyphae during growth. Fungal cellulases and hemicellulases have optimal activity at a low pH at high temperature (Heidorne *et al*., 2006). The detected xylose (and xylobiose) and arabinose most likely originate from hemicellulose and pectin while glucose originates from cellulose (Hatakka and Hammel, 2011; Mäkelä *et al*., 2015). Striking is also the differences in mannitol and trehalose in time that also differ between the two fungi. Both components are storage molecules and may play a role in water stress (Rast and Pfyffer, 1989; Fillinger *et al*., 2001). The differences between the two fungi in

mannitol and trehalose concentrations at the beginning of the storage is likely due to the differences in the amount of mycelium (Nayan *et al*., 2018). While for CS-AWS, mannitol remains fairly constant, this compound increases in concentration in LE-AWS during storage. It is unclear why this is only seen for *L. edodes.* Trehalose concentration decreased sharply after 2 weeks of storage and is likely due to metabolisation. Finally, the increase of glycerol is also remarkable especially in *C. subvermispora* treated AWS. Glycerol is a anaerobic degradation product of fatty acids. A higher concentration in *C. subvermispora* treated AWS might be partly explained by the chemical/enzymatic degradation of the ceriporic acids that are only present in this fungal species.

Despite the changes in the chemical composition of fungal WS during storage observed here, no significant changes were seen in the IVGP. The data presented here were accumulated gas production after 72-h. Especially the increase in free sugars, however, might lead to a faster utilization by rumen microbiota. Although the nutritional value as measured by the IVGP did not change, the generation of various organic compound and increase in titratable acidity may affect the palatability of the stored fungal treated AWS.

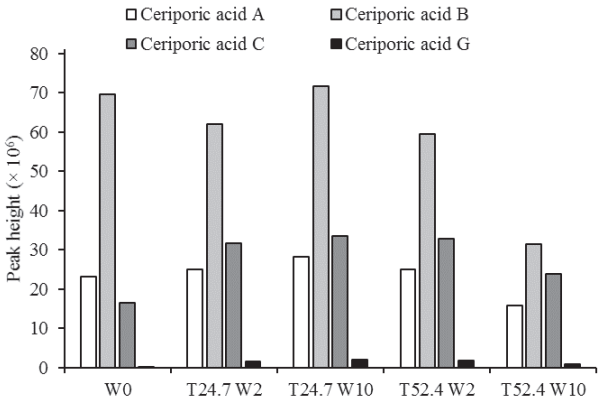

Fig. 4.5. Ceriporic acid A, B, C and G in *Ceriporiopsis subvermispora* treated wheat straw stored anaerobically at 24.7 °C (T24.7) and 52.4 °C (T52.4) for 2 (W2) and 10 (W10) weeks, measured by liquid chromatography mass spectrometry (LC-MS). W0 represent the start of anaerobic storage (AWS aerobically incubated with *C. subvermispora* at 25 °C for 7 weeks).

4.4 Conclusions

Wheat straw treated with the white-rot fungi *C. subvermispora* and *L. edodes* resulted in changes in chemical composition and colour, but not *in vitro* gas production when stored anaerobically. Especially acids and sugar monomers accumulated in time but the overall fibre composition remained relatively constant. Since the concentration of compounds measured by GC-MS analysis were semi-quantified, it might be that observed changes are quantitatively not significant and explain the retained nutritional value after prolonged anaerobic storage. The reason for this is that the main fraction of the fungal treated wheat straw is the nutritious fibre (64-67%) and that is mostly unaffected by storage. These finding have implications for the application of biological treatment of lignocellulosic waste streams.

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Chapter 5

Preference of fresh and stored *Ceriporiopsis subvermispora* and *Lentinula edodes* treated wheat straw by goats

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Abstract

The successful application of fungal treated lignocellulosic biomass as an animal feed ingredient depends on its acceptance by animals. The objective of this study was to test the preference of fresh and stored fungal treated wheat straw (WS) by non-lactating Saanen goats. Three studies were conducted. Wheat straw was aerobically treated with *Ceriporiopsis subvermispora* (CS) and *Lentinula edodes* (LE) for 7 weeks before being stored at -20 °C (fresh CS [CS_f], fresh LE [LE_f]) until use. The CS_f and LE_f were also anaerobically stored at 54 °C (stored CS [CS_s], stored LE [LE_s]) for 7 weeks before being frozen at -20 °C until use. In study 1, preference for individual feedstuffs was tested and included grass silage (GS), maize silage (MS), CS_f, LE_f and WS. Goat preference showed a ranking of GS > MS > CS_f = LE_f = WS, with goats avoiding CS_f, LE_f and WS in the 2 h preference test. Study 2 compared the preference of CS_f, LE_f and WS when included at 50% (as is basis) in a GS/MS based feed over 6 d. Goats had a higher ($P < 0.05$) intake rate of the CS_f and LE_f than the WS containing feed, with no significant difference observed between CS_f and LE_f. In study 3, the preference of goats between diets containing (50% as is basis) fresh and stored fungal treated WS was investigated. In an identical study design to study 2, the goats showed a higher intake rate for the CS_s and LE_s containing feeds. Wheat straw treated with *C. subvermispora* and *L. edodes* were significantly less preferred to GS and MS. This feeding study showed that fungal treated wheat straw can be used as a major part in a diet for goat and the storage increases its palatability.

Key words

Fresh; stored; fungal treated wheat straw; preference; goats.

5.1 Introduction

Annually, agricultural activities produce vast amounts of plant-derived residues that contain a relatively high lignin content (e.g. various straws, bagasse, oil palm fronds). Although abundant in cellulose and hemicellulose, these residues are to a limited extent used as animal feed due to their low fermentability as a result of the highly lignified structure of the cell walls (Van Kuijk *et al*., 2015a). De-lignifying white-rot fungi are capable of increasing the bioavailability of the cellulose and hemicellulose to rumen microbes by oxidising the lignin and thereby increasing the availability of cellulose and hemicellulose to rumen microbes. The increase in fermentability by rumen microbes of fungal treated wheat straw (WS) has been extensively studied on a laboratory scale (Tuyen *et al*., 2012; Van Kuijk *et al*., 2015b; Nayan *et al*., 2018). Among the tested white-rot fungi, *Ceriporiopsis subvermispora* and *Lentinula edodes* are the two most promising selective fungi for highly lignified biomass degradation (Tuyen *et al*., 2012). These two fungi produce numerous metabolites, including organic acid and reduced the pH during colonisation of the substrate (Aguiar *et al*., 2006; Kwak *et al*., 2016; Nayan *et al*., 2018). Recently it was shown (Mao *et al*., 2018) that *C. subvermispora* and *L. edodes* treated WS can be successfully stored without additives under anaerobic conditions at 20 °C up to 64 days. *L. edodes* is an edible mushroom and has the advantage that the mycelium is generally regarded as save (GRAS status). Little is known regarding the safety of consuming *C. subvermispora* as this fungus does not produce mushooms and is not used in products for human consumption. However, a study (Oguri *et al*., 2013) have investigated the improvement in digestibility of *C. subvermispora* treated bamboo to ruminants with no detrimental effects reported on the animals. Although no data are available on toxin production by *C. subvermispora*, this fungus does not produce any of 34 mycotoxins analysed (Chapter 3), indicating that they are safe for consumption by animals, at least from the perspective of the analysed mycotoxins.

As mentioned above, both *C. subvermispora* and *L. edodes* produce a multitude of organic acids to lower the pH of the substrate to 3.4-4.3 (Mao *et al*., 2018). Diet preference is important since it directly influences the utilization of fungal treated biomass by ruminants. The diet selection is influenced by a number of factors, which are either associated with the feedstuff or the animal, including chemical composition, texture and taste of the feed, past experience, post-ingestive consequences, fermentability, digestibility (Provenza *et al*., 1994; Morand-Fehr, 2003).

The main objective was to investigate the acceptance of fresh and stored *C. subvermispora* and *L. edodes* treated WS by adult goats. Three trials were conducted to determine the preference of goats between (1) fresh fungal treated and untreated WS relative to two common roughages grass silage and maize silage, (2) WS, *C. subvermispora* treated and *L. edodes* treated WS and (3) between fresh and stored *C. subvermispora* and *L. edodes* treated WS.

5.2 Materials and methods

5.2.1 Preparation of fungal strains and spawn

The fungi *C. subvermispora* (CBS 347.63; Origin: USA) and *L. edodes* (CCBAS389; Origin: Czech Republic), stored in liquid nitrogen from the collection of Plant Breeding (Wageningen University & Research, The Netherlands) were used. Strains were propagated on malt agar extract, containing 10 g l^{-1} malt extract (Oxoid Ltd, Hampshire, UK) and 17.5 g l^{-1} micro agar (Duchefa Biochemie B.V, Haarlem, The Netherlands) and incubated at 25 °C for one to two weeks (depending on the fungus) until the mycelium colonized the entire plate. Six pieces (approximately 1.5 - 2 cm^2) of colonized agar were transferred to 1.2 l (TP1200 + TPD1200; Combiness, Nevele, Belgium) boxes (n = 12) for each fungal species with ~ 400 g of pre-autoclaved sorghum grains, and incubated at 25 °C until the mycelium fully colonized the grains. The prepared spawn was stored at 4 °C until further use.

5.2.2 Preparation of fresh and stored fungal treated wheat straw

Wheat (*Triticum aestivum* L.) straw was filled in monofilament net bags (50 × 80 cm, pore size: 1.5 × 1.5 mm) and immerged in tap water for 3 days, after which the water was drained from the bags for a period of 5 h. Approximately 2 kg of wet WS per polypropylene zipper filter bag (Model: PP75-BEU6/X32-57; Sac O_2, Nevele, Belgium) was used to fill 68 bags which were autoclaved at 121 °C for 1 h. The bags were left at room temperature in a flow cabinet overnight, and aseptically inoculated with 50 g of *C. subvermispora* or *L. edodes* spawn. The bags were hermetically sealed with an electric sealer in a flow cabinet before the spawn and straw were thoroughly mixed and the bags were placed in a climate controlled room at 25 °C. Seven weeks later, bags for each fungal species (n = 24) were individually sealed in a plastic bag to prohibit loss of moisture during storage at -20 °C (fresh fungal treated WS). The remaining bags (10 bags for each

fungal species) were individually placed in two plastic bags and hermetically sealed after pressing air out by hand. The bags were heated in an oven set at a temperature of 55 °C for another 7 weeks, to simulate a long time storage after which the bags were stored at -20 °C (stored fungal treated WS). A data logger (EBI 300; Ebro, Ingolstadt, Germany) was placed in the oven to record the temperature at 1 h intervals during storage. The content of all the bags, containing either fresh or stored fungal treated WS, were combined per fungal species and thoroughly mixed using a peddle mixer (Ingeniørfirmaet Halvor Forberg AS, F60, Larvik, Norway) before again being stored (-20 °C) in separate bags containing ~ 670 g of material until used as a feed or to formulate diets.

5.2.3 Animal experiment

The study was exempt form an animal ethics approval as it was considered by the Wageningen University Authority for Animal Welfare to fall outside the criteria for the requirement of an animal experiment. The housing, care and experimental procedures were performed according to the rules of Wageningen University Authority for Animal Welfare.

5.2.3.1 Animals, housing and feeding

Six healthy non-lactating Saanen goats with a similar age of 1.5-2.5 yr and a body weight of 64.1 (SD ± 2.4 kg) were purchased from a local commercial farm. The goats were randomly assigned to six individual and adjacent pens (1.9 × 3.25 m) containing saw dust as bedding material. Animals were able to hear and see each other and neighbouring animals could have limited physical contact through the barred fences. Each pen was equipped with a 1.25 m plastic trough constructed of a half-pipe that was divided in five equal sized smaller sub-troughs (1 to 5) (l × w × h: 0.25 × 0.25 × 0.175 m). Each trough was covered by a non-transparent lid and located at a height of 0.53 m (top) from the floor. A separate water bowel was provided to allow goats free access to clean water. All goats were subjected to the same experimental procedures and received the same diets during the entire experiment. A video camera was positioned at one side of each pen with clear visibility of the trough to monitor the intake of the goats and remaining feed. The goats had *ad libitum* access to a basal diet comprised of (on a dry matter (DM) basis): 32.1% grass silage (GS), 32.1% maize silage (MS), 32.1% wet WS, 3.3% soybean meal and 0.4% pre-mix which was provided two times a day (10.00 and 17.00 h) throughout

the experiment with the exception of the training period and preference testing. This ration ensured that the energy and protein requirement of non-lactating goats (NRC, 2012) were met when fed *ad libitum*. The wet WS was produced by immerging WS in water overnight and draining for 5 h. Goats were familiarised to the housing, diet and management for 6 days after which the training started.

5.2.3.2 Training period

A 7 day training period was started to habituate the goats to the preference testing procedures. The basal diet was removed from the trough at 21:30 h every day to make animals moderately hungry. Each day, 600 g of a mixed diet [as is basis: 20% GS, 20% MS, 20% wet WS, 20% fresh *C. subvermispora* treated WS (CS_f) and 20% *L. edodes* treated WS (LE_f)] was equally distributed over 3 sub-troughs selected randomly per pen. At 9:00 h, the lid of each trough was removed providing goats access to the feed and at 10:00 h the lids were used to cover the troughs before feed residues were collected per sub-trough and weighed. From day 3 to 7, goats were additionally provided with 200 g wet WS in one randomly chosen sub-trough.

5.2.3.3 Ranking experiment

The six goats were used to determine the preference among individual feedstuffs (LE_f, GS, WS, MS and CS_f) with 200 g of each feedstuff placed in sub-through 1 to 5, respectively on day 1. Intake rate (expressed as g h^{-1}) over the 1 h period was determined from the video cameras and determination of feedstuff refusals. The following day, the most preferred feedstuff of day 1 was no longer provided and after 1 h, intake rate was determined. On day 3, the three least preferred feedstuffs were provided to the goats and intake rate was determined over a 2 h period. The feedstuffs were always provided in the same sub-through.

5.2.3.4 Preference of diets containing CS_f, LE_f and WS

A 1 h preference test of diets containing the least preferred feedstuffs (CS_f, LE_f and WS) was performed immediately after the ranking experiment. The CS_f, LE_f and WS feedstuffs were mixed (50% as is basis) with a compound feed (CCS_f, CLE_f, and CWS, respectively) comprised of (DM basis): 48.15% GS, 48.15% MS, 3.3% soybean meal and 0.4% pre-mix. The CLE_f, CWS and CCS_f feeds (400 g of each) were provided to the goats in the

same sub-troughs as in the ranking experiment (1, 3 and 5, respectively) on day 1. The order of the three feeds was changed subsequent days so that each feed appeared in the same sub-trough twice in the six day experiment. Feed refusals were measured each day and intake rate was determined.

5.2.3.5 Preference of diets containing fresh and stored fungal treated WS

The day after the test to determine the preference of diets containing CS_f, LE_f and WS, the identical trial setup was used to determine the preference between CS_f and stored *C. subvermispora* treated WS (CS_s) when included at 50% (as is basis) in the compound feed. The CS_s and CS_f containing feed (600 g of each) were provided to the goats in sub-troughs 1 and 5, respectively on day 1. The order was changed subsequent days so that each compound feed appeared in the same sub-trough three times in the six day experiment. The identical trial setup was then used to determine the preference between diets containing fresh (LE_f) and stored *L. edodes* treated WS (LE_s).

5.2.4 Chemical composition and pH

Samples were collected and air dried in an oven at 70 °C until constant weight. The dried samples were ground in a hammer mill over an 1 mm sieve (100 AN; Peppink, Olst, The Netherlands) for analysis of residual dry matter, ash, nitrogen (N), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL). Residual dry matter was measured by placing samples at 103 °C for 4 h as described in ISO 6496 (1999), where after samples were incinerated in a muffle furnace at 550 °C for 3 h (ISO 5984, 2002). The N content was determined by the Kjeldahl method (ISO 5983, 2005) and crude protein was calculated as N × 6.25. Neutral detergent fibre, ADF and ADL were determined as described by Van Soest *et al*. (1991) using an Ankom fiber analyzer (A2000I; ANKOM Technology, Macedon, NY, USA). Cellulose was calculated as the difference between ADF and ADL, while hemicellulose was considered to be the difference between NDF and ADF.

For pH measurement, 30 g of fresh sample was weighed in a stomacher bag to which 270 ml demineralised water was added. The suspension was mixed in a stomacher (400 Circulator; Seward, Worthing, UK) at 230 rpm for 5 min, where after pH was measured using a calibrated pH meter (Model HI 9024; Hanna instruments, IJsselstein, The Netherlands).

5.2.5 Statistical analysis

The intake rate and intake percentage were analysed with the mixed procedure in SAS 9.3 using the model:

$Y_{ijkl} = \mu + F_i + D_j + (F \times D)_{ij} + G_k + \omega_{ijkl}$

where Y_{ijkl} was the dependent variable, μ the overall mean, F_i the fixed effect of feedstuff, D_j the fixed effect of day, $(F \times D)_{ij}$ the interaction between feedstuff and day, G_k the random effect of goat and ω_{ijkl} the error term. The Tukey test was used to compare effects and interactions that showed significant differences in intake rate and intake percentage. A linear regression model was fitted between the independent variable day and dependent variable average daily intake rate of fresh and stored CS and LE. Significance was declared at $P < 0.05$.

5.3 Results

The goats remained healthy throughout the experiment. During the 7 days of the training periods all goats adapted well to the feed containing CS_f and LE_f. There was no significant difference in average daily intake rate of the mixed diet when provided in sub-trough 1 to 5 (136.5, 124.3, 123.0, 133.8, 138.3 g h^{-1}, respectively) for any of the goats over the last 5 days of the training period. Regardless of the position of wet WS, this feedstuff was generally avoided (max daily intake rate 0.03 g h^{-1}) by all goats during the 5 days it was provided.

5.3.1 Chemical components and pH of individual feedstuffs

Table 5.1 shows the chemical composition and pH of individual feedstuffs offered to the goats throughout the experiments. The DM content was highest for GS (50.9%), followed by MS (35.4%), and the lowest DM content was observed for WS and fungal treated WS (18-20%). The hemicellulose and ADL content decreased significantly while ash, crude protein increased after *C. subvermispora* and *L. edodes* treatment of the WS for 7 weeks. The cellulose content decreased only slightly. The anaerobic storage at 54 °C for 7 weeks resulted in a decrease in hemicellulose and an increase in ADL. The ranking of cellulose + hemicellulose was: WS > LE_f > LE_s > CS_f > CS_s > GS > MS. The highest ADL content was observed for WS. Except for soybean meal, the crude protein content was ranked as

Table 5.1. Analysed composition and pH of feedstuffs used in the goat preference studies.

Ingredient	DM	Ash	Crude protein	Cellulose	Hemicellulose	ADL	pH
	g kg^{-1} as is	g kg^{-1} DM					
Grass silage	509.4	100.1	200.2	231.8	212.8	10.8	5.20
Maize silage	353.8	35.3	67.9	174.2	140.6	8.3	3.68
Wheat straw	198.5	32.8	31.4	491.7	326.1	73.7	7.52
CS treated (fresh[1])	179.6	65.3	52.7	467.4	138.2	18.3	3.55
CS treated (stored[2])	193.1	71.0	53.3	469.1	80.1	37.8	3.12
LE treated (fresh[1])	191.6	58.1	47.0	469.3	200.0	39.4	3.97
LE treated (stored[2])	200.0	66.7	48.1	475.8	149.2	48.3	3.42
Soybean meal	913.0	71.7	471.9	106.4	58.2	0.0	-

DM: dry matter; ADL: acid determined lignin; CS: *Ceriporiopsis subvermispora*; LE: *Lentinula edodes*.

[1] Conserved under anaerobic conditions for 7 weeks at -20 °C.

[2] Anaerobically stored for 7 weeks at 54 °C.

GS > MS > CS_f = CS_s > LE_f = LE_s > WS. The pH of WS was decreased by the *C. subvermispora* and *L. edodes* treatment to 3.55 and 3.97 after 7 weeks, and further lowered to 3.12 and 3.42, respectively with storage at 54 °C for an additional 7 weeks.

5.3.2 Ranking experiment

The intake rate of LE_f, GS, WS, MS and CS_f are present in Fig. 5.1. A strong preference of GS was exhibited by all goats on day 1, with an average intake rate of 766.0 g h^{-1}.

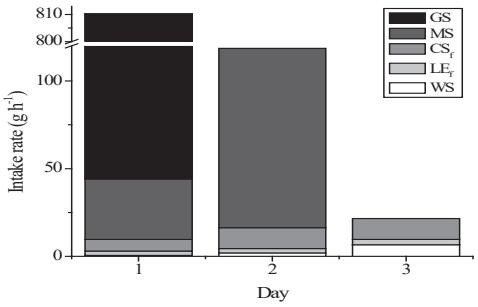

Fig. 5.1. Intake rate of grass silage (GS), maize silage (MS), wheat straw (WS), fresh *Ceriporiopsis subvermispora* treated WS (CS_f) and fresh *Lentinula edodes* treated WS (LE_f) when presented to goats (n = 6) at the same time during 3 consecutive days. Most preferred feedstuff was not provided to the goats the next day.

Much lower intake rates were found for MS (34.5 g h^{-1}), CS_f (6.7 g h^{-1}), LE_f (2.5 g h^{-1}) and WS (0.5 g h^{-1}). The intake rate of MS was increased (102 g h^{-1}) after the removal of GS on day 2, with still minor amounts of CS_f (12.0 g h^{-1}), LE_f (2.5 g h^{-1}) and WS (2.0 g h^{-1}) consumed by goats. After the removal of GS and MS and an extended consumption time of 2 h on day 3, small amounts of CS_f (11.9 g h^{-1}), LE_f (3.0 g h^{-1}) and WS (6.7 g h^{-1}) were consumed. The preference can be ranked as GS > MS > CS_f = LE_f = WS.

5.3.3 Preference of feed containing CS_f, LE_f and WS

Since goats avoided to consume significant amounts of CS_f, LE_f and WS, these feedstuffs were mixed with 50% of a compound feed comprised of GS, MS, soybean meal and a premix. Fig. 5.2 shows the average daily intake rate and percentage intake of CCS_f, CLE_f and CWS by goats over 6 days. The average daily intake rate of the three compound feeds by the 6 goats was 712.3 g h^{-1}, in which CCS_f, CLE_f and CWS accounted for 36.8% (262.7 g h^{-1}), 37.6% (267.5 g h^{-1}) and 25.6% (182.1 g h^{-1}). The intake rate and percentage intake of CCS_f and CLE_f was significantly higher compared to CWS ($P < 0.01$). There was no difference ($P = 0.978$) in the amount of CCS_f and CLE_f consumed by the goats.

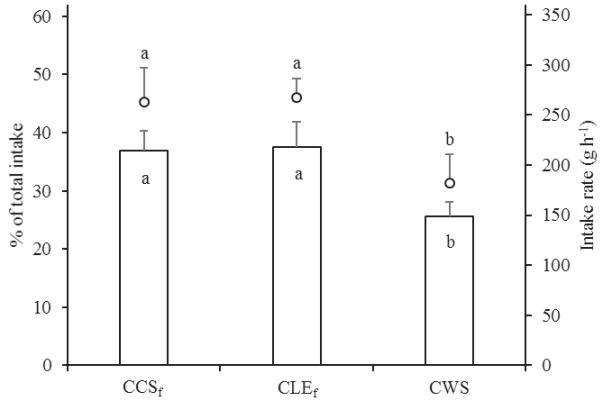

Fig. 5.2. Mean daily intake percentage (bars) and rate (○) of compound feeds containing (50% as is basis) either wheat straw (CWS), fresh *Ceriporiopsis subvermispora* treated WS (CCS_f) or fresh *Lentinula edodes* treated WS (CLE_f) of goats (n = 6) when presented at the same time during 6 consecutive days. Error bars are standard deviations. Means with different letters are significantly different ($P < 0.05$).

5.3.4 Preference of diets containing fresh and stored fungal treated WS

The difference in daily intake rate and intake percentage between CCS_f and CCS_s as well as CLE_f and CLE_s are shown in Fig. 5.3. The average daily intake rate of *C. subvermispora* treated WS (fresh + stored) was 830.6 g h^{-1}, comprising of 46.5% of CCS_f (387.7 g h^{-1}) and 53.5% CCS_s (443.0 g h^{-1}). The difference in daily intake rate ($P = 0.033$) and percentage intake ($P = 0.043$) between CCS_f and CCS_s was significant. For *L. edodes* treated WS (fresh + stored), the average daily intake rate (933.8 g h^{-1}) was higher than for the *C. subvermispora* treated WS containing compound feed. On average, goats consumed 43.0% CLE_f (412.4 g h^{-1}) and 57.0% CLE_s (521.4 g h^{-1}) per day with the difference being significant ($P < 0.001$). The daily intake rate of CCS_f remained stable ($P > 0.05$) from day 1 to 6, while a consistent linear increase ($P < 0.05$) of CS_s was observed, with an average daily intake rate of 316.3 g h^{-1} on day 1 to 499.6 g h^{-1} on day 6. A similar increase also occurred for the CLE_s which consistently increased in a linear fashion ($P < 0.05$) from day 1 (488.6 g h^{-1}) to 6 (548.7 g h^{-1}). The daily intake rate of CLE_f showed a non-significant change over the 6 days.

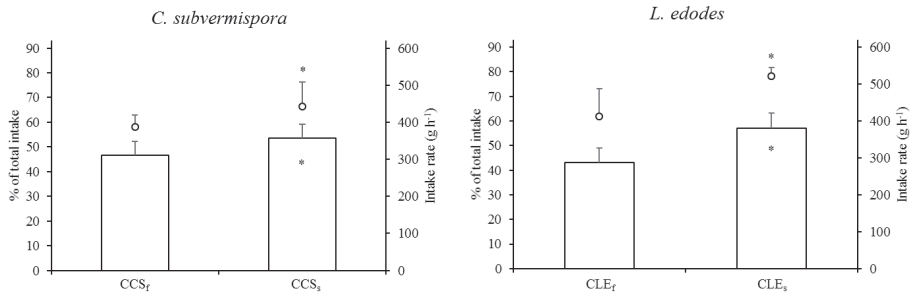

Fig. 5.3. Mean daily intake percentage (bars) and rate (○) of compound feeds containing (50% as is basis) either fresh or stored *Ceriporiopsis subvermispora* treated WS (CCS_f or CCS_s), fresh or stored *Lentinula edodes* treated WS (CLE_f or CLE_s) of goats (n = 6) when presented at the same time during 6 consecutive days. Error bars are standard deviations. * indicates significant ($P < 0.05$) difference between means.

5.4 Discussion

The chemical composition of WS and fungal treated WS are in line with previous studies (Van Kuijk *et al*., 2015b; Mao *et al*., 2018; Nayan *et al*., 2018), showing that hemicellulose and ADL were significantly decreased in the fungal treated WS without a significant reduction in cellulose. The reduction in pH caused by *C. subvermispora* and *L. edodes* indicates that the WS was acidified through the production of organic acids by these fungi. The reduced pH is comparable with results of Mao *et al*. (2018), showing that a *C. subvermispora* treatment decreased the pH of wheat straw to 3.66 while *L. edodes* decreased the pH to 4.24 after 7 weeks of aerobic incubation. Although the crude protein content (Table 5.1) showed an increase after the fungal treatment, the absolute amount of crude protein did not change as nitrogen from substrates (Van Kuijk *et al*., 2015b) is assimilated by white-rot fungi and not lost as volatile compounds. Storage of the fungal treated WS for 7 weeks resulted in a further decrease in pH by 0.43 and 0.55 units for *C. subvermispora* and *L. edodes*. The decrease in pH is likely the result of chemical reactions occurring during storage at high temperature. The decrease in content of hemicellulose is likely due to the continuing activity of hemicellulases resulting in solubilisation of hemicellulose and, as a consequence, a relative increase in ADL. Although the two fungi were inactivated due to the temperature (54 °C) (Zervakis *et al*., 2001) and anaerobic conditions, some enzymatic activity may remain and chemical reactions are likely to occur between metabolites or other components at this elevated temperature. The increase in ADL might also be partly due to the repolymerisation of lignin fragments (Li *et al*., 2007).

The current study design was able to observe clear daily intake rate differences among the feedstuffs tested. Goats who showed the greatest preference for GS, were more reluctant to consume MS while avoiding CS_f, LE_f and WS, even when the preference time was extended for the latter three feedstuffs (Fig. 5.1). Daily intake rate is dependent on a number of factors including, level of hunger, past experience, hedonic value, feedstuff composition, novelty of feedstuffs and learned aversion (Provenza *et al*., 1994; Baumont *et al*., 2000; Morand-Fehr, 2003). The goats used in the present study were familiar with GS, MS and WS through previous consumption on farm. Rejection or reluctance to consume novel feeds is a behavioural strategy of animals to avoid the consequences of ingesting toxins (Provenza *et al*., 2003). Although goats did not have access to the fungal treated WS before the experiment, they were familiarised with it as part of the mixed diet

during the 7 day training period. Therefore, the very limited intake of CS_f and LE_f observed appears to indicate an avoidance of the fungal treated WS due to sensory aspects (pH, taste, texture and aroma) rather than an effect of novelty. Indeed CS_f and LE_f have a strong and specific odor and contain different organic acids (Chapter 3). The avoidance of CS_f and LE_f by goats can also be related to the diverse secondary metabolites produced from the bioconversion of biomass by *C. subvermispora* and *L. edodes*. Regardless of the liberation of sugars, the break-down of the lignin and linkages between cell wall components result in the accumulation of aromatic compounds and organic acids (Aguiar *et al*., 2006; Kwak *et al*., 2016; Van Kuijk *et al*., 2017). Oxalic acid also produced by these fungi (Aguiar *et al*., 2006; Kwak *et al*., 2016) might influence the rumen microbiota hence influence feedstuff preference (Duncan *et al*., 1997; Duncan *et al*., 2000). Post-ingestive consequences are not likely an explanation for individual feedstuff preference in the present study as minor intakes of CS_f and LE_f were observed although sensory properties might have allowed the goats to anticipate post-ingestive consequences (Baumont *et al*., 2000).

The difference in preference between CS_f, LE_f and WS became clear when mixed with 50% of a GS/MS based diet. Both CCS_f and CLE_f were significantly more consumed compared to CWS (Fig. 5.2). Although a different fungal strain was used, Shrivastava *et al*. (2012) observed a numerically higher DM intake per unit body weight (18.8 vs 17.9 g) of a *Ganoderma sp*. rckk02 treated compared to an untreated WS diet by goats. The dilution effect of negative and addition of positive sensory components are responsible for the higher intake rates as the other variables were kept constant (e.g. time, feeding regime, housing conditions). As also observed when feeding the basal diet, inclusion of fungal treated biomass in a compound feed is a good strategy to increase the intake of this feed ingredient by goats. Besides the sensory characteristics of the fungal treated material, the high intake rate of CCS_f and CLE_f compared to CWS might also be because of the poor nutritional value of WS. No difference in daily intake rate between CCS_f and CLE_f was observed, even though in previous studies (Tuyen *et al*., 2012; Mao *et al*., 2018), the *in vitro* degradation of CS_f was higher than LE_f.

The preference experiment between fresh and stored fungal treated WS was also conducted utilizing the compound feed containing 50% of GS/MS. The actual storage temperature measured by the data logger over the 7 weeks of storage was 54 °C. The results indicate that the goats preferred, for both fungal species, the stored WS (Fig. 5.3),

which indicates that storage at high temperature clearly changes the chemical composition and thus influences taste. Previously also a decrease in hemicellulose and increase in ADL with storage at elevated temperature (52.4 °C) was observed, but no significant effect was seen on the *in vitro* degradation in rumen fluid of CS_s and LE_s (Chapter 4). Chemical analyses have shown that a number of sugars, organic acids and other compounds produced by *C. subvermispora* and *L. edodes* accumulated with storage. In the current study, hemicellulose was also found to decrease and ADL to increase and it is likely that a number of metabolites (e.g. sugars, organic acids) were present in the stored compared to the fresh fungal treated WS.

Over the 6 days, the goats increased their daily intake of the compound feed containing the stored fungal treated WS compared to the fresh. It can be speculated that the formed components during storage increased the palatability. Goats have receptors for the five primary taste modalities of salt, sweet, sour, bitter and umami (Ginane *et al*., 2011). As mentioned before, chemical reactions are likely to have occurred during the storage for 7 weeks at 54 °C which may have affected the palatability by decreasing unpalatable or generating palatability enhancing components. Potentially goats may have learned through post-ingestive effects and increase the intake of the stored fungal treated WS. It has been reported that rumen microbial degradation of certain plant secondary compounds can increase after a period of adaptation, and results in the preference pattern of ruminant animal (Duncan *et al*., 2000).

5.5 Conclusions

Direct consumption of fungal (*C. subvermispora* and *L. edodes*) treated wheat straw by goats is low compared to grass silage and maize silage. When included in a compound feed containing 50% grass and maize silage, goats showed a clear preference for straw treated with *C. subvermispora* and *L. edodes* over wheat straw. When the same compound feed contained fungal treated wheat straw stored at 54 °C for 7 weeks, fresh fungal treated wheat straw was less preferred. Treating wheat straw with *C. subvermispora* and *L. edodes* increases the palatability of wheat straw for goats when included at 50% in a compound feed with storage improving the preference.

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Chapter 6

Wheat bran addition improves *Ceriporiopsis subvermispora* and *Lentinula edodes* growth on wheat straw but not delignification

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Abstract

A rapid and complete colonization of lignocellulosic biomass by white-rot fungi is a prerequisite for an efficient and contamination free delignification. To improve colonization, mycelium growth and delignification by *Ceriporiopsis subvermispora* and *Lentinula edodes* of wheat straw, with addition of 2, 4, 6 and 10% of wheat bran for 0, 2, 4, 8 and 12 weeks, was evaluated. The ergosterol content, chemical composition and *in vitro* gas production (IVGP) by ruminal microorganisms were measured. The fungal biomass and starch amount was significantly ($P < 0.001$) affected by the inclusion level of wheat bran added and incubation time. An increase in added wheat bran increased fungal biomass with different effects observed for the two fungi. The added starch from the wheat bran was degraded by *C. subvermispora*, mainly during the first two weeks (up to 44%), whereas, *L. edodes* degraded starch during 12 weeks up to 83%. The supplementation of wheat bran, however, did not affect ($P > 0.05$) the amount of hemicellulose and lignin at the end of the incubation. There was no significant effect of wheat bran addition on the IVGP for the two fungal treatments. Supplementation of wheat straw with wheat bran results in a quicker fungal colonization, which is useful for *C. subvermispora*, a fungus that forms only thin mycelium and is more prone to contamination than *L. edodes*.

Key words

Ceriporiopsis subvermispora; *Lentinula edodes*; wheat bran; fibre composition; starch.

6.1 Introduction

White-rot fungi are the most effective microbiota in decaying wood in nature. They generate a mixture of oxidative enzymes, such as peroxidases and laccases to degrade lignin, obtaining in this way access to the polysaccharides hemicellulose and cellulose (Wan and Li, 2012; Pollegioni *et al*., 2015). Selective white-rot fungi are characterized by the degradation of lignin and hemicellulose during the initial phase of colonizing lignocellulosic biomass, with less or no degradation of cellulose (Wan and Li, 2012). This property makes these organisms highly suitable to upgrade lignocellulosic biomass to feed for ruminants. To achieve a successful bioconversion process by white-rot fungi, a fast and complete colonization of the biomass is a prerequisite, because of the high risk of contamination during the initial incubation period. Growth and delignification vary substantially with fungal species and substrates (Tuyen *et al*., 2012, 2013). In reported studies, it requires several weeks of incubation with *C. subvermispora* and *L. edodes* to obtain a desirable delignification and optimum in rumen fermentability of wheat straw (Tuyen *et al*., 2012; Nayan *et al*., 2017; Mao *et al*., 2018). The carbohydrates from agricultural biomass can serve as the carbon and energy source for growth of the fungi. Fungi can grow under low nitrogen (N), but an increase of some limited nutrients might have a positive effect on the initial growth of the fungi (Van Kuijk *et al*. 2016a). Wheat bran, a by-product of the wheat milling process, is usually used as feed for animals. It is also used to increase the production of a number of edible mushroom species (Kurt and Buyukalaca, 2010), indicating that this supplement might well enhance mycelium growth. Wheat bran consists of hemicellulose, protein, cellulose, lignin and other minor compounds (Maes and Delcour, 2001; Brijwani *et al*., 2010). Sometimes wheat bran can be high in starch. Hemicellulose and starch can be a source of carbon, while protein can be a N resource for protein synthesis by fungi.

The aim of this study was to determine the effect of supplementation of wheat straw with different amounts of wheat bran on the time required to fully colonize the substrate and its effect on selectivity and extent of lignin degradation by *Ceriporiopsis subvermispora* and *Lentinula edodes*.

6.2 Materials and methods

6.2.1 Fungal strain and inocula preparation

The fungi *Ceriporiopsis subvermispora* (CBS 347.63; USA) and *Lentinula edodes* (CCBAS389; Czech Republic), maintained in liquid N at the collection of Plant Breeding (Wageningen University & Research, The Netherlands), were used. The strains were propagated on new malt extract agar plates containing 10 g l^{-1} malt extract (Oxoid Ltd, Hampshire, UK) and 17.5 g l^{-1} micro agar (Duchefa Biochemie B.V, Haarlem, The Netherlands) at 25 °C until the mycelium fully colonized the surface. Two colonized agar pieces (~ 1.5 cm^2) of each fungal species were transferred to plastic boxes (Model: OS60 + OD60; Combiness, Nevele, Belgium) with ~ 75 g pre-autoclaved sorghum grains and incubated at 25 °C for 4-5 weeks (depending on the fungus), until the mycelium fully covered each grain. The prepared spawn was stored at 4 °C for further use.

6.2.2 Fermented materials and solid culture

Organic wheat straw was cut, using a chopping machine (Pierret Industries, Corbion, Belgium), to lengths of approximately 0.5-2.0 cm. The straw was used to fill monofilament net bags (50 × 80 cm, pore size: 1.5 × 1.5 mm) and immersed in water for three days before water was drained for 5 h. The wet straw was collected, mixed by hand and distributed into 1.2 l boxes containing a filter (Model: TP1200 + TPD1200; Combiness, Nevele, Belgium). The wheat straw was replaced with 0, 2, 4, 6 and 10% (w/w dry matter) of wheat bran and thoroughly mixed. The total wet weight in each container was 200 g with water added to account for the difference in dry matter (DM) between the wheat straw and bran. All the containers were autoclaved at 121 °C for 1 h and cooled to room temperature in a flow cabinet overnight. Each box was aseptically inoculated with 5 g of *C. subvermispora* or *L. edodes* spawn, and the boxes were placed in a climate controlled room at 25 °C for 2, 4, 8 and 12 weeks. Three boxes of each wheat bran addition were collected each week and stored in a freezer for further freeze drying. The weight of the boxes was recorded to calculate the absolute amount and DM loss during the fungal treatment.

6.2.3 Analytical methods

6.2.3.1 Chemical composition analysis

The content of the boxes was freeze dried and ground to pass a 1 mm sieve (100 AN; Peppink, Olst, The Netherlands) for analysis of DM, ash, N, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL). The samples were dried at

103 °C for 4 h for DM analysis (ISO 6496, 1999), and incinerated in a muffle furnace at 550 °C for 3 h to determine the ash content (ISO 5984, 2002). The N content was analysed by the Kjeldahl method as described by ISO 5983 (2005). Starch content were measured according to ISO 15914 (2004). Both detergent fibres and ADL were analysed according the methods described by Van Soest *et al*. (1991), using an Ankom fibre analyzer (A2000I; ANKOM Technology, Macedon, NY, USA). Cellulose content was calculated as the difference between ADF and ADL, while the hemicellulose content was the difference between NDF and ADF. The absolute amount was calculated per box and DM loss of the boxes after a fungal treatment was calculated in comparison with wheat straw with added spawn without incubation.

6.2.3.2 Ergosterol analysis

The ergosterol content was determined by an HPLC method as described by Niemenmaa *et al*. (2008). Briefly, approximately 0.2 g of freeze dried substrate was weighed into glass tubes and 3 ml of KOH (in methanol) was added to each tube. The suspension was thoroughly mixed and saponified in a heating block at 80 °C for 60 min. After cooling the tube to room temperature, 20 µl of cholecalciferol (vitamin D_3, 0.5 µg μl^{-1}) was added as an internal standard, after which the extraction process with 1 ml of water and 2 ml of hexane was performed twice, with 10 min shaking and 10 min centrifuging (4000 rpm) each time. The hexane layer was transferred to a new glass tube and dried under vacuum at 30 °C for 60 min. The sterol residue was dissolved in 1 ml of methanol and mixed briefly before filtered into an inserted glass vial for HPLC analysis. The samples were analysed using an HPLC with a Waters HPLC-PDA system (Alliance HPLC system, Milford, USA), Ergosterol was detected at 280 nm.

6.2.3.3 *In vitro* gas production

The *in vitro* gas production was performed as described by Cone *et al*. (1996). In short, rumen fluid was collected from three lactating, rumen fistulated cows, fed *ad libitum* corn silage and grass silage. The combined rumen fluid was filtered through two layers of cheese cloth and mixed with a mineral buffer solution. All procedures were conducted under continuous flushing with CO_2. An accurately weighed amount (approximately 0.5 g) of sample was incubated in 60 ml buffered rumen fluid for 72 h and gas production

was recorded automatically. Gas productions were corrected for blank gas productions, i.e. gas production by buffered rumen fluid without sample.

6.2.4 Statistical analyses

The analysis of ergosterol content, chemical composition and IVGP were performed using the generalized linear model with SAS 9.3. Significant differences were identified using the model:

$Y_{ijk} = \mu + IL_i + T_j + IL_iT_j + \omega_{ijk}$

where Y_{ijk} is the response variable *ijk*, μ is the overall mean, IL_i is the effect of the wheat bran inclusion level *i*, T_j is the effect of time *j*, IL_iT_j is the interaction between inclusion level *i* and time *j* and ω_{ijk} the error term. The difference in DM loss per unit of fungal biomass formed between the 10% of wheat bran addition and no addition was also performed using the generalized linear model with SAS 9.3, and the model:

$Y_{ik} = \mu + IL_i + \omega_{ik}$

where Y_{ik} is the response variable *ik,* μ is the overall mean, IL_i is the effect of the wheat bran inclusion level *i* and ω_{ijk} the error term. Tukey's test was used for multiple comparisons, and significance was declared as $P < 0.05$. The regression between ergosterol content and DM loss was performed in SAS 9.3, and R^2 (coefficient of determination) and P values were recorded. Probability values < 5% were set to be significant with a trend between 5 and < 10%.

6.3 Results

6.3.1 Fungal growth on wheat straw with different inclusion levels of wheat bran

Ergosterol was measured as an indicator for fungal biomass formation. *C. subvermispora* and *L. edodes* exhibited a different growth pattern during the 12 weeks of incubation (Fig. 6.1A and B, respectively). The ergosterol content at week 0 for both fungal treatments was 27.8-36.8 µg g^{-1} dry substrate. *C. subvermispora* showed a relatively rapid increase in biomass up to week 8, where after was remained constant. At 8 weeks, the ergosterol content was 139 (0%), 159 (2%), 190 (4%), 200 (6%) and 238 (10%) µg g^{-1} dry substrate. The growth of *L. edodes* showed a clear lag phase in the first two weeks of incubation and an increase in biomass was seen between 2 to 12 weeks, with a maximum content of ergosterol of 404 (0%), 455 (2%), 532 (4%), 588 (6%) and 745 (10%) µg g^{-1} dry substrate

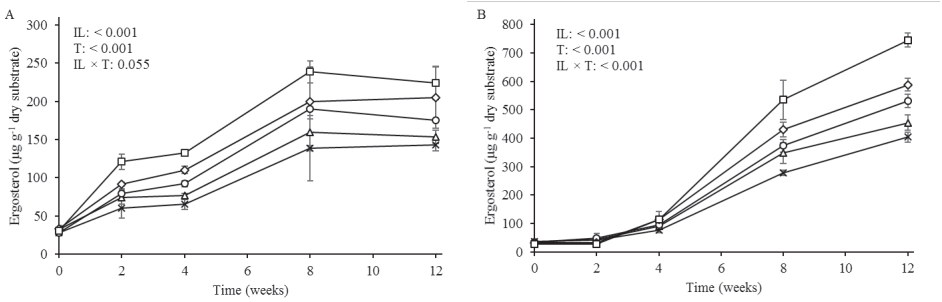

Fig. 6.1. Ergosterol content of (A) *Ceriporiopsis subvermispora* and (B) *Lentinula edodes*, grown on wheat straw with different inclusion levels [IL: 0% (×), 2% (Δ), 4% (○), 6% (◊) and 10% (□)] of wheat bran at different incubation times (T: 0, 2, 4, 8 and 12 weeks).

at week 12. The content of ergosterol was significantly affected ($P < 0.001$) by the wheat bran inclusion level and incubation time ($P < 0.001$) of the two fungal treatments. There was a trend for an interaction ($P = 0.055$) between wheat bran inclusion level and incubation time for the *C. subvermispora* treatment and a significant interaction for *L. edodes*.

6.3.2 Chemical composition

Compared to wheat straw, wheat bran had a higher content of starch and N, but lower contents of cellulose and ADL (lignin), whereas the hemicellulose content was comparable to that of wheat straw (Table 6.1). Changes in the amount of selected components (per 200 g of starting material) of *C. subvermispora* and *L. edodes* treated wheat straw are shown in Fig. 6.2 and 6.3. As expected, with an increasing inclusion level of wheat bran, the amount of N and starch increased at week 0 (Fig. 6.2). As expected, DM loss was continually increasing over 12 weeks of incubation with the two fungi (Fig. 6.2). *C. subvermispora* degraded starch almost exclusively during the first 2 weeks (up to 44%) and at 12 weeks, 0.78-1.60 g of the initial starch was degraded, which is equivalent

Table 6.1. Chemical composition (g kg^{-1} dry matter) of wheat straw and wheat bran.

Wheat	Ash	Nitrogen	Starch	Cellulose	Hemicellulose	ADL
Straw	40.6	3.1	11.7	482.4	271.8	71.3
Bran	51.5	25.2	272.2	83.6	264.6	22.4

ADL: acid detergent lignin.

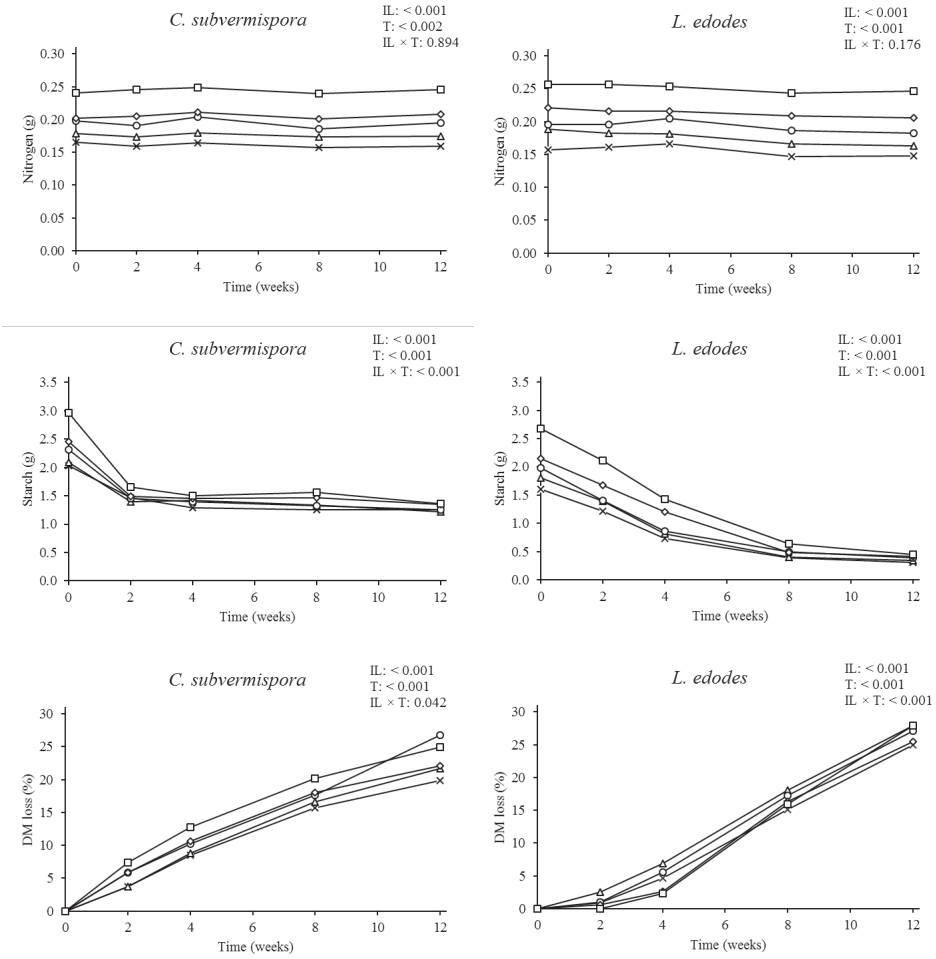

Fig. 6.2. Amount of nitrogen and starch (per 200 g starting material), and dry matter (DM) loss of *Ceriporiopsis subvermispora* and *Lentinula edodes*, grown on wheat straw with different inclusion levels [IL: 0% (×), 2% (Δ), 4% (○), 6% (◊) and 10% (□)] of wheat bran at different incubation times (T: 0, 2, 4, 8 and 12 weeks).

to a degradation of 38-54%. For *L. edodes*, a continuous starch degradation was seen over the 12 weeks with 80-83% of the starch degraded (1.30-2.23 g) at week 12. The absolute amount of cellulose and hemicellulose decreased with increasing incubation time (Fig. 6.3). *C. subvermispora* degraded lignin mainly from week 2 to 8, with a reduction of 77-82% at week 8. *L. edodes* degraded lignin mainly from week 4 to the end of the incubation,

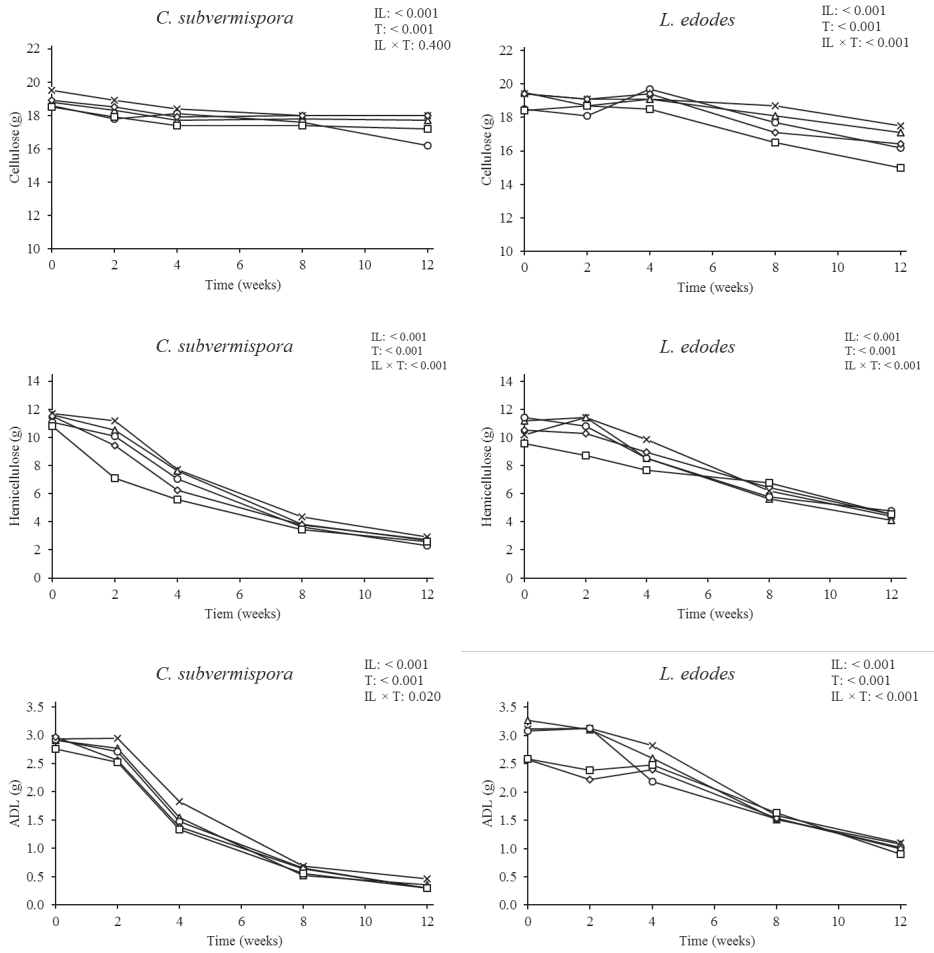

Fig. 6.3. Fibre composition and acid detergent lignin (ADL) content (per 200 g starting material) of *Ceriporiopsis subvermispora* and *Lentinula edodes*, grown on wheat straw with different inclusion levels [IL: 0% (×), 2% (Δ), 4% (○), 6% (◊) and 10% (□)] of wheat bran at different incubation times (T: 0, 2, 4, 8 and 12 weeks).

with a reduction of 61-67% at week 12. During the 12 weeks of incubation, inclusion level of wheat bran ($P < 0.001$) and incubation time significantly affected the content of N, starch, DM loss, cellulose, hemicellulose and ADL in the two fungal treatments. No interaction was found between wheat bran inclusion level and incubation time with N content in the two fungal treatments, neither of cellulose content in the *C. subvermispora* treatment. No statistical difference in starch content between inclusion levels of wheat bran at week 12 in the two fungal treatments was observed. The hemicellulose and ADL

content at week 0 of all inclusion levels of wheat bran showed no significant difference, neither at week 12 in the *C. subvermispora* treatment. For *L. edodes*, although variation in inclusion level of wheat bran at week 0 was observed, no significant difference in hemicellulose and ADL at week 12 were observed.

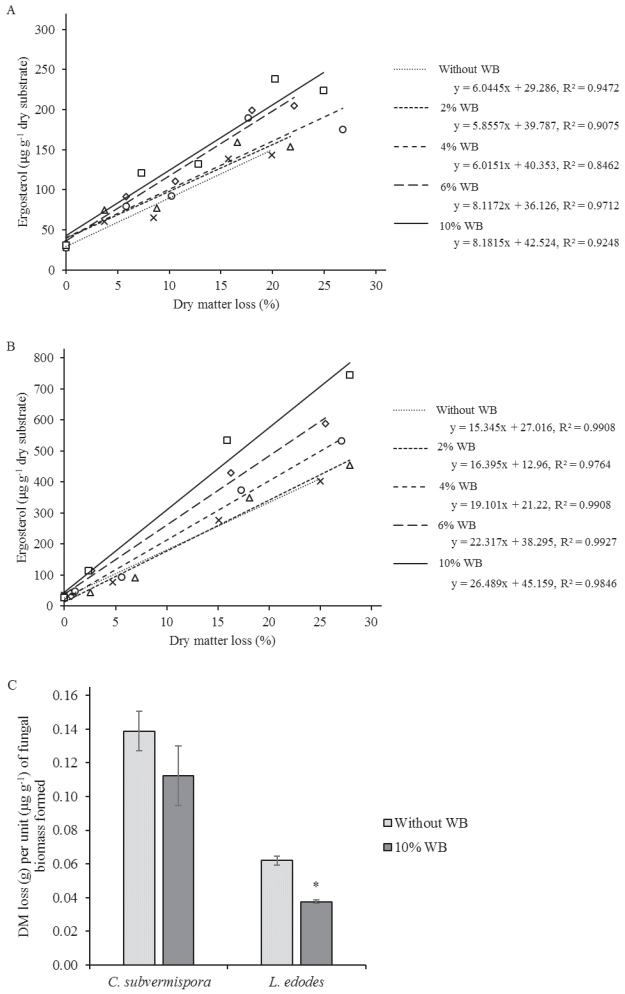

Fig. 6.4. Linear regression of ergosterol content and dry matter (DM) loss of (A) *Ceriporiopsis subvermispora* and (B) *Lentinula edodes*, grown on wheat straw with different inclusion levels [IL: 0% (×), 2% (Δ), 4% (○), 6% (◊) and 10% (□)] of wheat bran (WB) at 0, 2, 4, 8 and 12 weeks. All the regression equations were significant ($P < 0.05$). (C) The DM loss (g) per unit (µg g^{-1}) of ergosterol formed with 10% added and without WB at 12 weeks. * indicates significant ($P < 0.05$) differences, with corresponding "without WB" value. Error bars are standard deviations.

6.3.3 Relationship between ergosterol content and DM loss

As expected, strong linear regressions were observed between the ergosterol content and the DM loss with the various wheat bran additions for both *C. subvermispora* and *L. edodes* treatments (Fig. 6.4A and B, respectively). For both fungi, but especially for *L. edodes*, an increase in the slope of the fitted curve was seen upon an increase in the amount of wheat bran added (Fig. 6.4A and B). The DM loss per unit of ergosterol formed after 12 weeks was higher with 0 than with 10% wheat bran added in the two fungal treatments, but only reached significance ($P < 0.001$) in the *L. edodes* treatment (Fig. 6.4C). This value is numerically higher in the *C. subvermispora* treatment than the *L. edodes* treatment, either without or with the addition of wheat bran.

6.3.4 *In vitro* gas production (IVGP)

All the treatments showed a similar pattern of IVGP from week 0 to week 12 (Fig. 6.5). A decrease in IVGP was seen within 2 weeks of incubation with *C. subvermispora* and within 4 weeks of incubation with *L. edodes*. Only incubation time significantly ($P < 0.001$) affected the IVGP for the two fungal treated wheat straws.

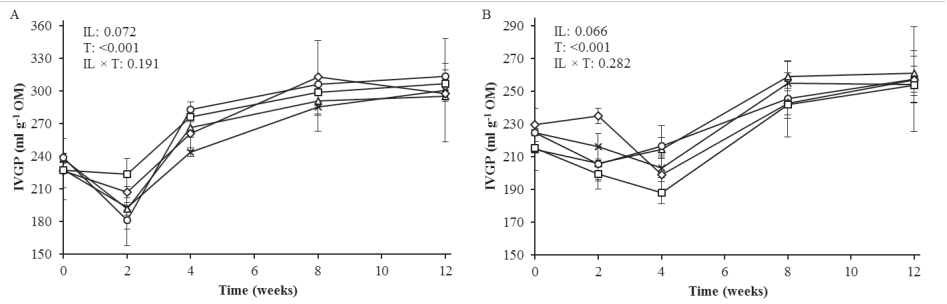

Fig. 6.5. Cumulative (72h) *in vitro* gas production (IVGP) of (A) *Ceriporiopsis subvermispora* and (B) *Lentinula edodes*, grown on wheat straw with different inclusion levels [IL: 0% (×), 2% (Δ), 4% (○), 6% (◊) and 10% (□)] of wheat bran at different incubation times (T: 0, 2, 4, 8 and 12 weeks).

6.4 Discussion

Sterilization (121 °C for 1 h) of wheat straw on a larger scale is costly and alternatives, such as pasteurizing wheat straw at a lower temperature (70 °C), may be an alternative. This approach works well when inoculation occurs with *L. edodes*, but results often in contaminations with *C. subvermispora* (Ratni *et al*., unpublished observations). Microscopic examinations of large batches of pasteurized wheat straw showed that only straw parts not colonized by *C. subvermispora* showed infections (Sonnenberg, unpublished observation). Compared to *L. edodes*, *C. subvermispora* produces very thin mycelium. An improvement of the colonization degree by increasing the amount of mycelium might thus protect wheat straw from infections. The initial ergosterol content of the wheat straw (week 0) likely originates from fungi present on the wheat straw and from the spawn. The supplementation of wheat straw with wheat bran had a major positive effect on the amount of *C. subvermispora* mycelium formed. From visual observations, the addition of 10% of wheat bran showed an amount of fungal biomass after 2 weeks that was similar to incubation after 8 weeks without addition of wheat bran (data not shown). In *L. edodes* mushroom cultivation, often green moulds are seen on substrates, but in most cases they are overgrown by *L. edodes*, with no or hardly any effect on mushroom yields (personal observations by mushroom growers). This indicates that this fungus is less sensitive to infections than *C. subvermispora*. Although the addition of wheat bran at different inclusion levels also had a positive effect on the growth of *L. edodes*, it visually inhibited the colonization speed of this fungus above 6% of wheat bran added (data not shown). This led to a patchy growth leaving large parts of the substrate for a long period uncolonized and thus prone to infections. The addition of wheat bran also did not decrease the lag time of growth of *L. edodes*. The addition of wheat bran to lignocellulose wastes can also offer a cheap alternative for the expensive sterilization. By mixing cassava waste (mainly peels) with wheat or rice bran, temperatures up to 73 °C can be reached by natural fermentation and proved to be a good substrate for oyster mushroom cultivation without any sterilization (Sonnenberg *et al*., 2015). The linear regression between DM loss and fungal biomass indicates a more efficient substrate utilisation (conversion of substrate into fungal biomass) of the two fungi with an increasing inclusion level of wheat bran. On average, for every unit of wheat bran added, the increase in growth was 117% for *L. edodes* and only 27% for *C. subvermispora*. The significantly lower DM loss per unit of fungal biomass of wheat straw, to which 10% of

wheat bran was added, compared to wheat straw treated with *L. edodes* (Fig. 6.4C) also indicates that DM was more efficiently converted to fungal biomass with the addition of 10% of wheat bran. Although no significant difference was detected for the *C. subvermispora* treatment, this fungus also showed a similar pattern. The difference between the two fungi might indicate a difference in substrate utilisation efficiency. Lashermes *et al*. (2016) also saw a difference in efficiency of bioconversion (here named Carbon Use Efficiency, CUE) when *Phanerocheate chrysosporium* was grown on different parts of maize plants. This different bioconversion efficiency was suggested to depend on the investment needed in enzyme synthesis and enzyme efficiency on substrates.

A difference was seen between the two fungi on how starch was degraded. *C. subvermispora* degraded starch mainly during the first 2 weeks, whereas *L. edodes* showed a continuous degradation during 12 weeks. *L. edodes* also degraded more starch than *C. subvermispora*, which might partly explain the larger increase in biomass for this fungus. It is also interesting that both fungi degrade starch to the same extent, independent of the amount of wheat bran added. The difference in utilization of starch from the millet grains by *C. subvermispora* and *L. edodes* was reported by Van Kuijk *et al*. (2016b), showing that *C. subvermispora* degrades very little starch, while *L. edodes* significantly decreased the starch content after five weeks of colonization. The difference in starch utilisation between *C. subvermispora* and *L. edodes* might partly be due to differences in enzyme types and numbers involved in starch degradation. For *C. subvermispora*, for example, only four alpha-amylase genes are identified in the genome whereas *L. edodes* contains twelve of these genes (https://genome.jgi.doe.gov/).

The addition of wheat bran appears to not greatly enhance lignin degradation by *C. subvermispora* and *L. edodes* grown on wheat straw. This might partly be explained by the effect of the added wheat bran on the MnP enzyme activity of the fungi (Silva *et al*., 2005). Amirta *et al*. (2006) found a significant higher weight loss and faster lignin reduction of Japanese cedar wood supplemented with wheat bran, cultured by *C. subvermispora*, than with no addition. The difference with the present study might be due to the different substrate and differences in chemical composition of the wheat bran used. Different batches of wheat bran might vary in the amount of starch. Striking is also that the absolute amount of hemicellulose and ADL at the end of the incubation is very similar for all inclusion levels of wheat bran, although different amounts of fungal biomass was

formed. *C. subvermispora* clearly degraded more hemicellulose and lignin than *L. edodes*, as reported in previous studies (Tuyen *et al*., 2012, Nayan *et al*., 2018). The different pattern in ADL degradation might be explained by the differences in the underlying delignification mechanism of wheat straw (Van Erven *et al*., 2018). For both fungi, an increased degradation of ADL and hemicellulose might reflect the influence of chemical bonds between lignin and hemicellulose (Hatakka and Hammel, 2011), i.e. degradation of one component leads to solubilisation of the other component.

The IVGP of wheat straw was increased after the fungal treatment as described by many studies (Tuyen *et al*., 2012; Nayan *et al*., 2017). The initial decrease in IVGP is likely due to the degradation of easily accessible sugars (mainly originating from starch) by the fungi. This decrease almost coincided with the starch degradation pattern by both fungi: *C. subvermispora* rapidly degraded starch only during the first two weeks, whereas *L. edodes* degraded starch in a more continuous manner to 12 weeks. It is surprising to see that, regardless of the inclusion levels of wheat bran added, both fungi degraded starch, hemicellulose and ADL to almost the same level and as a consequence, the IVGP profiles were relatively similar for the different amounts of wheat bran added.

6.5 Conclusions

Although the present work shows that addition of wheat bran does not improve selective delignification of wheat straw, it does improve the colonization rate, especially of *C. subvermispora* and might, therefore, reduce the risk of contaminations during the delignification of biomass for animal feeds when this fungus is used.

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Chapter 7

General discussion

7.1 Outline of the thesis

Selective white-rot fungi can be highly effective in improving the nutritive value of biomass high in lignocellulose. By degrading lignin and its linkages with carbohydrates (cellulose and hemicellulose) the fungi improve the anaerobic degradation by rumen microbes (Sharma and Arora, 2015; Rouches *et al*., 2016). Bioconversion of lignocellulosic biomass by white-rot fungi has been extensively researched at a laboratory scale. For wheat straw, a significant increase in fermentability by rumen microbes has been observed after treatment by the fungi *Ceriporiopsis subvermispora* and *Lentinula edodes* (Tuyen *et al*., 2012; Van Kuijk *et al*., 2015a; Nayan *et al*., 2018). Also the nutritive value of other lignocellulosic biomass (e.g. bamboo, sugarcane bagasse, wood, oil palm fronds) has been shown to be able to be improved by a fungal treatment (Okano *et al*., 2009; Rahman *et al*., 2011; Tuyen *et al*., 2013; Van Kuijk *et al*., 2015a). In general, selective fungi require several weeks to colonize the biomass, simultaneously degrading significant amounts of lignin and hemicellulose, but little cellulose. The longer fungal growth on biomass occurs, the more loss of dry matter (DM) and polysaccharides is observed (Van Kuijk *et al*., 2015a). It is, therefore, important to terminate the fungal treatment at the most optimal time and subsequently use a method to effectively conserve the fungal treated substrate for prolonged periods of time. In this thesis, *C. subvermispora* and *L. edodes* treated wheat straw were successfully stored at 20 °C for 64 days (Chapter 2) and at elevated temperatures, up to 52.4 °C for 10 weeks (Chapter 4). Nutrient composition and feeding value remained stable without the need for additives. A series of organic acids, sugars and other compounds are released after the fungal treatment (Chapter 3), some of these compounds accumulate further during storage (Chapter 4). Although further safety testing on animals is needed, the findings in this thesis indicate that fungal treated wheat straw is safe for animals to consume: none of the analysed mycotoxins (n = 34) were detected (Chapter 3) and goats consuming the treated wheat straw in a mixed diet remained healthy throughout the 28 day study (Chapter 5). The palatability of stored fungal treated wheat straw was shown to be higher than that of fresh treated straw, as reflected by the higher intake by the goats (Chapter 5). In order to reduce the risk of contamination by other fungi during colonization, the supplementation of wheat bran in wheat straw was studied and found to improve the colonization of *C. subvermispora* (Chapter 6). The results in this thesis provide important information for

the use of fresh and stored fungal treated wheat straw as an animal feed ingredient in practice.

7.2 The advantages of fungal acidification of the substrate

The pH of wheat straw was lower than 6 after three days of soaking in water, indicating that some fermentation may have occurred and organic acids were produced under the low oxygen conditions in water (Chapter 2 and 4). Unfortunately, organic acids in dry and wet wheat straw were not analysed by GC-MS (Chapter 3 and 4). In comparison, the pH of wheat straw was close to neutral (pH 7.52) when immerged in water overnight (Chapter 5) (Fig. 7.1). This shows that soaking length influenced the initial pH of the biomass. The buffering capacity of dried wheat straw, however, was very low and minor amounts of acid resulted in a relatively large change in pH indicating the presence of a low concentration of hydrogen ions. Most filamentous fungi prefer a slight acidic growth environment (Magnuson and Lasure, 2004), and are able to adapt their gene expression to the environmental pH (Peñalva *et al*., 2008). Decreasing the initial pH might be favourable for fungal growth but how large the influence of the initial acidity is on fungal growth, remains to be determined. Chapter 2, 3, 4 and 5 showed that *C. subvermispora* and *L. edodes* acidified wheat straw during the colonization, as reported by Nayan *et al*. (2018). In Chapter 3 and 4 it was shown that a number of organic acids were produced

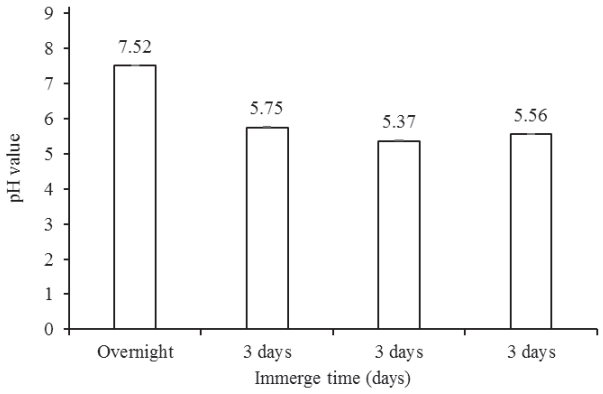

Fig. 7.1. pH of wheat straw immerged in water overnight (Chapter 5) and for three days (Chapter 2 and 4).

by the fungi. One widely accepted advantage of fungal acidification is that in this way the contamination by undesirable microbiota is prevented (Magnuson and Lasure, 2004). This might not only be caused by a low pH, but also by the antibacterial properties of some organic acids (Kwak *et al*., 2016). Therefore, identifying the organic acids, released by *C. subvermispora* and *L. edodes* is important. The natural acidification by *C. subvermispora* and *L. edodes* can be compared to ensiling and will greatly contribute to the conservation during storage (Chapter 2 and 4).

The enzymes involved in lignin degradation produced by *C. subvermispora* and *L. edodes* include manganese peroxidases (MnPs) and laccases (Hatvani and Mécs, 2002; De Souza-Cruz *et al*., 2004; Fernández-Fueyo *et al*., 2012; Nayan *et al*., 2017). Although MnPs and laccases play an important role in lignin degradation, their activity does not always correlate well with lignin loss in the substrate (Aguiar *et al*., 2006; Nayan *et al*., 2017) indicating the complexity of lignin degradation and the limited knowledge we still have of the complete tool box of fungi. MnP oxidizes Mn^{2+} to the highly reactive Mn^{3+} which is stabilized by chelation with short-chain organic acids, such as oxalic acid. Chelated Mn^{3+} can diffuse into the dense cell wall structures high in lignin and attack lignin molecules, resulting in the formation of instable free radicals causing the spontaneous disintegration of lignin (Hofrichter, 2002). Numerous organic acids were measured in freeze dried samples in Chapter 3 and 4. It is possible that some volatile compounds, including organic acids were lost during the freeze drying procedure employed for analyses. Unlike other white-rot fungi, *C. subvermispora* is able to produce a series of specific itaconic acids, so called ceriporic acids (A-H) that play an important role in lignin depolymerisation (Enoki *et al*., 2002; Amirta *et al*., 2003; Nishimura *et al*., 2008, 2012a, b). In Chapter 3, ceriporic acids A, B, C and G were found to accumulate in *C. subvermispora* treated wheat straw. Ceriporic acid B is proposed to suppress cellulose depolymerisation (Rahmawati *et al*., 2005), while ceriporic acid G facilitates a more efficient lignin degradation (Nishimura *et al*., 2012b). It needs to be noted that other ceriporic acids, which were not detected in Chapter 3 and 4, might also exist. These ceriporic acids might have additional properties due to their specific structural characteristics. In fresh fungal treated wheat straw, acetic acid was the main volatile fatty acid detected in all test samples, as is seen in silages from grass or maize. No propionic, isobutyric and butyric acid were detected, and only very minor amounts of isovaleric and

valeric acid, close to the detection limit, were detected in several samples (n = 6) (Chapter 2).

7.3 Changes in wheat straw caused by white-rot fungi

Since fungal growth and lignin degradation vary between fungal species/strains for different types of biomass (Tuyen *et al*., 2012, 2013; Nayan *et al*., 2018), it is important to select the correct fungal species and strain for a specific biomass. *C. subvermispora* (CBS 347.63; USA) and *L. edodes* (CCBAS389; Czech Republic) are two of the most effective white-rot fungi among the species and strains tested on wheat straw at Wageningen University (Tuyen *et al*., 2012; Nayan *et al*., 2018). These strains were thus also used in the research reported in this thesis. Conventional wheat straw (Chapter 2 and 5) and organic wheat straw (Chapter 3, 4 and 6) collected locally were used as substrate in this thesis. Fig. 7.2 summarizes the chemical composition of the wheat straw and autoclaved wheat straw as analysed in Chapters 2-6. The untreated (soaked, non-autoclaved) regular wheat straw contained 25.8-52.7, 3.6-5.0, 474-492, 303-326 and 69-79 g kg^{-1} DM of ash, nitrogen, cellulose, hemicellulose and acid detergent lignin (ADL) (Chapter 2 and 5), showing that some variation existed in the substrates used between chapters and even within a chapter (Chapter 2), when the same batch of wheat straw was used. The organic wheat straw (soaked, non-autoclaved) contained (g kg^{-1} DM) 36.1 ash, 2.4 nitrogen, 481.4 cellulose, 284.2 hemicellulose and 71.4 ADL (Chapter 4). Regardless

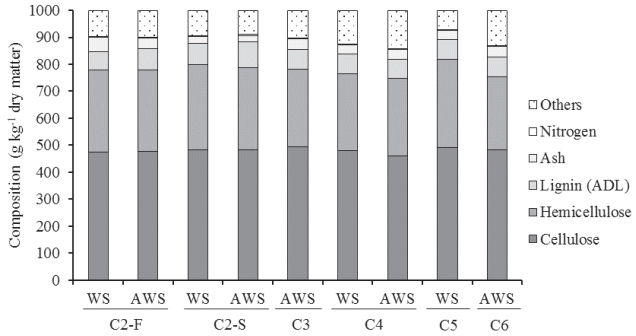

Fig. 7.2. Chemical composition of untreated wheat straw (WS, soaked) and autoclaved wheat straw (AWS) as determined in different chapters (C). Chapter 2 includes a fungal treatment (F) and a storage (S) experiment.

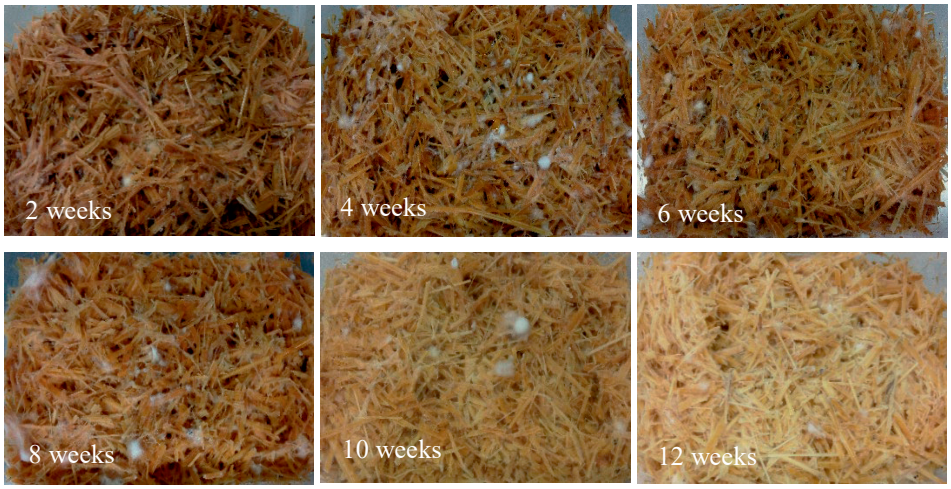

Fig. 7.3. Colour change of wheat straw after colonization with *Ceriporiopsis subvermispora* for 2 to 12 weeks.

of the type of wheat straw, the chemical composition was comparable between experiments. The one hour autoclaving also had a minor effect on the chemical composition (Chapter 2 and 4) of the wheat straw.

Autoclaving of wheat straw, however, resulted in a lower 'L' (darkness) and a higher 'a' (redness) value (before autoclaving: 'L': 75.8, 'a': 0.77, 'b': 19.6; after autoclaving: 'L': 70.6, 'a': 2.03, 'b': 19.5). Incubating autoclaved wheat straw under aerobic conditions for a period of 7 weeks hardly affected the 'L', 'a' and 'b' values ('L': 69.7, 'a': 2.13, 'b': 19.8). Bekhta and Niemz (2003) showed that thermal treatment (heating between 100 and 150 °C) darkens wet spruce wood by lowering 'L' and increasing the 'a' value. A gradual colour change is seen during the colonization of fungi on autoclaved wheat straw up to 12 weeks, especially by *C. subvermispora* (Fig. 7.3) (Chapter 6). *C. subvermispora* and *L. edodes*, caused the substrate to become brighter, yellower and redder after 7 weeks of treatment (Chapter 4), similar to what is observed to occur with many other fungi (Arora *et al*., 2011). The changes in colour during a fungal treatment might possibly be used as a simple indicator and potentially a rapid quality control parameter for predicting the extent of mycelium colonization and lignin degradation. The breakdown of cell wall structures "softens" the wheat straw, and allows for more *C. subvermispora* treated and *L. edodes* treated wheat straw (regular and organic) to be packed in 0.5 l glass jars than

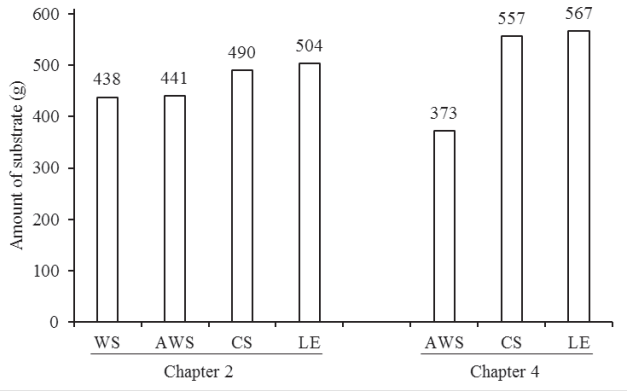

Fig. 7.4. Weight of wheat straw (WS), autoclaved wheat straw (AWS), *Ceriporiopsis subvermispora* (CS) treated AWS and *Lentinula edodes* (LE) treated AWS in 0.5 l jars in Chapter 2 and 4. There were 54 glass jars for each substrate in Chapter 2 and 61 jars in Chapter 4.

autoclaved and non-autoclaved wheat straw (Fig. 7.4) (Chapter 2 and 4). The softness might partly contribute to the higher intake of *C. subvermispora* and *L. edodes* treated wheat straw by goats, than moistened wheat straw in a mixed diet (Chapter 5).

The loss of weight of the biomass with fungal treatment provides an estimation of organic matter degradation. After 8 weeks of treatment, approximately 16% (Chapter 3 and 6) DM loss in wheat straw treated with *C. subvermispora* and 15-18% with *L. edodes* was observed, with the DM loss continuing with a longer incubation period (Chapter 6; Van Kuijk *et al*., 2015a). The greatest loss occurred for hemicellulose and ADL. *C. subvermispora* only caused 6.8-7.8% cellulose loss, while 62.6-65.2% and 72.0-76.8% loss of hemicellulose and ADL was observed after 8 weeks, respectively (Chapter 3 and 6). A similar pattern occurred with the *L. edodes* treatment, with only 3.6-4.5% cellulose loss, but 38.8-52.8% and 46.7-49.3% loss of hemicellulose and ADL, respectively. I have to point out here that hemicellulose was measured according to the gravimetric method of Van Soest (1991), where residues (insoluble) are determined. A substantial part of the "loss" of hemicellulose will be due to solubilisation and not by catabolism by the fungus (Nayan *et al*., 2019). Solubilisation of hemicellulose is partly due to hemicellulases excreted by the fungus, but also due to the degradation of lignin which is bound to hemicellulose (Nishimura *et al*. 2018). The minor degradation of cellulose is a common characteristic of selective white-rot fungi (Martínez *et al*., 2011). A number of common water soluble sugars are detected after a fungal treatment (Chapter 3 and 4). Although the

data in Chapter 3 and 4 provide information on what compounds are produced, there is not always a clear explanation why some are formed.

7.4 Storage of fungal treated wheat straw

The optimum lignin degradation by white-rot fungi may take several weeks to months, depending on the fungal species, strain, substrate and environmental conditions (Van Kuijk *et al*., 2015b; Rouches *et al*., 2016). It is important to terminate the fungal activity at the optimum time to yield the highest nutritional value, so it can be used as a feed ingredient.

In practice, lignocellulosic biomass is often produced in large quantities in a short period of time during harvest (e.g. rice, wheat and oil palm fronds). For efficiency purposes, the production of fungal treated biomass is best to also occur in large quantities. Once produced, the majority of the biomass would then require storage/conservation for extended periods of time (months) as feeding of the fungal treated biomass will occur in small quantities over a long period of time. In addition, storage is important to enable feed material to be available during periods of low feed availability (e.g. winter, dry season). Preserving fungal treated wheat straw at low temperatures (like spawn stored at 4 °C) can slow down the activity of the fungi, but it requires freezing facilities and energy input and hence is not applicable under practical circumstances. In the current thesis, the storage of fungal treated wheat straw was conducted by mimicking the ensiling process, which is normally used for preserving fresh forages, such as grass and maize. The difference between the storage of fungal treated wheat straw and the usual ensiling process of forages are summarized in Table 7.1. A successful ensiling process of forages mainly depends on the presence of an anaerobic environment, and a rapid decrease of the pH at the start of the process by naturally present or added lactic acid bacteria, which convert water soluble carbohydrates to predominantly lactic acid. Unlike ensiling, the acidification properties by *C. subvermispora* and *L. edodes* during the aerobic incubation appears to be sufficient to allow *C. subvermispora* and *L. edodes* treated wheat straw to be successfully stored under anaerobic conditions (jars, Chapter 2 and 4; plastic bags, Chapter 5). Some oxygen remaining in the fungi treated biomass will be removed, as the remaining oxygen is further used by the fungi until strict anaerobic conditions are achieved. White-rot fungi are aerobic microorganisms which will become inactive when

Table 7.1. Comparison of the storage of fungal treated wheat straw and the ensiling process of forages.

Phase	Condition	Fungal treatment of wheat straw	Ensiling of forages
Pre-treatment	Microbiota	• White-rot fungi	-
	Required treatment	• Chopping	• Air/sun drying
		• Soaking	• Chopping
		• Sterilization	
		• Aerobic incubation	
	Output (in a range)	• Increased *in vitro* digestibility	-
		• Moderate loss of polysaccharides	
		• Acidify biomass	
		• Change in organic acid, sugars and other compounds of substrate	
	Optimum length	• 5-12 of weeks (in this thesis)	-
Storage	Microbiota	• White-rot fungi, maybe minor amounts of other microbiota	• Lactic acid bacteria and other microbiota
	Growth	• Anaerobic conditions (oxygen consumed by fungi)	• Anaerobic conditions (oxygen consumed by plant respiration and by aerobic microbiota)
		• No or very slow growth	• Fast initial growth then no growth
		• Storage temperature has little effect on the growth of fungi	• Storage temperature affects growth of lactic acid bacteria
	Output (in a range)	• Stable *in vitro* digestibility	• Stable or moderate decrease *in vitro* digestibility
		• Moderate loss of polysaccharides	• Moderate loss of polysaccharides
		• Stable or slightly lower pH	• pH decrease and then stable
		• Further acidification of biomass	• Acidification of biomass
		• Accumulation of some organic acids, soluble sugars and other compounds	• Accumulation of organic acids (mainly lactic acid) and then stable
	Duration	• 10 weeks (this thesis), maybe longer	• Depends on storage condition (e.g. temperature)

oxygen levels are low (Nsolomo *et al*., 2000; Pavarina and Durrant, 2002).

Regardless of the storage temperature, fungal treated wheat straw further acidifies without a significant decrease in cellulose with storage under anaerobic conditions (Chapter 2 and 4). Addition of lactic acid bacteria and molasses at the beginning of the storage did not affect the pH and titratable acidity of *C. subvermispora* treated wheat straw (Chapter 2). For *L. edodes* treated wheat straw, addition of lactic acid bacteria and molasses lowered the pH to a value similar to *C. subvermispora* treated wheat straw (Chapter 2). The additional drop likely occurred by lactic acid produced by the added lactic acid bacteria and molasses. This shows that some anaerobic and acid tolerant microorganisms, such as lactic acid bacteria are able to survive on the substrates acidified by some white-rot fungi after a period of adaption. This might specifically occur with fungi that have a lower acidification capability. The acidification of biomass depends on the characteristics of the fungi and the buffer capacity of the substrates. In the case that some white-rot fungi are not able to acidify the biomass during colonization (Zadražil, 1977; Yang *et al*., 2001) or the biomass has a high buffering capacity, additives can be an efficient way to prohibit the growth of contaminating bacteria, as described by Yang *et al*. (2001). Ensiling is a commonly used method for the conservation of fresh crops e.g. grass and maize. The agricultural biomass used for a fungal treatment is typically dry material that can be stored easily (e.g. straws and bagasse), enabling aerobic fermentation to be performed all year round, and storage can be conducted under a wide range of climatic conditions. In Chapter 4 it is reported that a storage temperature of up to 52.4 °C for 10 weeks did not affect the *in vitro* gas production, even though some changes in hemicellulose and ADL occurred. This indicates that storage temperature (within a range) might not be a key factor influencing the nutrient stability of fungal treated wheat straw, as with the ensiling process. The latter is because the temperature will have little effect on inactive white-rot fungi compared to the active lactic acid bacteria required to be active under a anaerobic environment during the ensiling process. This is an important advantage for the storage of fungal treated wheat straw during warm seasons in tropical countries.

7.5 Fungal treated wheat straw as animal feed

Safety is a primary concern when it comes to fungal treated wheat straw as a feedstuff for animals. *L. edodes* is a common, edible and medical fungus, while *C. subvermispora* does not produce fruit bodies, and as such, is not used for consumption. In Chapter 3, none of

the 34 analysed mycotoxins were detected in organic wheat straw and wheat straw treated for 8 weeks with *C. subvermispora* and *L. edodes*, indicating these fungi are safe for animals to consume, at least from the perspective of the analysed mycotoxins. However, it is still possible that some specific, hitherto unknown, mycotoxins are synthesized by *C. subvermispora* and *L. edodes*, or that toxic products are generated especially during the degradation of lignin. During the degradation of lignocellulose by white-rot fungi, free aromatic compounds, such as free-hydroxyl phenols, can be produced (Mäkelä *et al*., 2015). These phenolic compounds can be toxic to fungi and also to most other microorganisms, such as those residing in the rumen (Borneman *et al*., 1986; Theodorou *et al*., 1987). White-rot fungi have a series of pathways to convert these compounds to less or non-toxic compounds. Depending on the concentration in the biomass, the amount of biomass consumed and the species, these formed compounds may exert effects on the animal. However, the increase in *in vitro* gas production (Chapter 2, 4 and 6) and the acceptance by goats (Chapter 5) indicate that *C. subvermispora* and *L. edodes* treated wheat straw is safe for consumption by ruminants.

The reduction of mycotoxins by white-rot fungi can be an additional benefit, next to the increase in fermentability of the biomass. Some white-rot fungi are able to detoxify certain mycotoxins (Wang *et al*., 2011; Yehia, 2014; Das *et al*., 2015; Branà *et al*., 2017). The results in Chapter 3 do not provide support for the detoxification of mycotoxins by *C. subvermispora* and *L. edodes*, as the levels of mycotoxins in the autoclaved organic wheat straw were below the detection limits. Alternariol, beauvericin, deoxynivalenol, enniatin A1, B and B1, nivalenol and zearalenone were present, however, on regular wheat straw (Table 7.2). This was unexpected because fungicides are usually used on regular wheat straw, but not on organic wheat straw. It might be interesting to study the detoxification effects of *C. subvermispora* and *L. edodes*, because mycotoxins can be produced from harvest to storage of agricultural residues.

7.6 The acceptance of fungal treated wheat straw by ruminant animals

The acceptance of fungal treated wheat straw by animals is important since this directly determines the amount of substrate they consume. In Chapter 5, the goats avoided *C. subvermispora* and *L. edodes* treated wheat straw, as well as wheat straw, even though the *in vitro* degradation of fungal treated wheat straw was higher than wheat straw

Table 7.2. Mycotoxins presented on regular wheat straw.

Mycotoxin	Amount (mg kg^{-1} dry sample)
15-Acetyl-deoxynivalenol	< 0.2
3-Acetyl-deoxynivalenol	< 0.08
Aflatoxin B1	< 0.0025
Aflatoxin B2, Aflatoxin G1, Agroclavine, Roquefortine C	< 0.0025
Aflatoxin G2, Alternariol-methylether	< 0.005
Alternariol	0.36
Beauvericin	0.063
Citrinin, Enniatin A, Mycophenolic acid, Nitropropionic acid	< 0.05
Deoxynivalenol	0.38
Deoxynivalenol-3-Glucoside	< 0.25
Diacetoxyscirpenol	< 0.0125
Enniatin A1	0.086*
Enniatin B	0.54
Enniatin B1	0.20
Fumonisin B1	< 0.1
Fumonisin B2	< 0.04
Fumonisin B3, HT-2 toxin, Penicillic acid, T-2 Toxin	< 0.02
Moniliformin	< 0.125
Nivalenol	0.13
Ochratoxin A	< 0.002
Sterigmatocystin	< 0.001
Zearalenone	0.20
α-Zearalenol, ß-Zearalenol	< 0.01

* Means mycotoxin content in another sample <0.05.

The detection limit of Alternariol, Beauvericin, Deoxynivalenol, Enniatin A1, Enniatin B, Enniatin B1 were < 0.02, < 0.025, < 0.2, < 0.05, < 0.05, < 0.05.

(Chapter 2, 4 and 6). Within the testing procedures, it is likely that palatability was the driving factor for their choice. Goats did not select the feed ingredients according to the available nutrients as they were able to obtain sufficient maintenance energy and protein from the basal diet (mainly grass silage and maize silage) which was provided after the preference test. The goats more easily accepted the fungal treated wheat straw as part of a mixed diet. Several changes are likely to have contributed to this increased acceptance when the fungal treated wheat straw was mixed with commonly consumed forages in the

Netherlands: (1) altered smell and taste (2) dilution of compounds formed in the fungal treated wheat straw and (3) lower moisture content from the higher DM content of grass silage and maize silage. As such, it is recommended to feed goats with fungal treated wheat straw as part of a mixed diet, although goats may more readily consume less palatable feeds with an increasing level of hunger. In Chapter 5, goats were supplied with *C. subvermispora* or *L. edodes* treated wheat straw mixed with 50% (fresh basis) of a grass and maize silage based diet. Diet selection behaviour of animals is also based on the salience of the flavours of the substrates included in the meal (Provenza *et al*., 1994). As such, an increased concentration of fungal treated wheat straw in a mixed diet might affect the acceptance and intake by an animal. Further investigations are, therefore, warranted to determine the acceptance of diets varying in the level of fungal treated wheat straw by goats as well as other ruminants. In addition, the acceptance of fungal treated wheat straw in a mixed diet by goats in Chapter 5 was conducted using non-lactating Saanen goats with past experience of the basal diet ingredients (wheat straw, grass silage and maize silage). Whether other goat breeds and goats with different past nutritional experiences will behave similarly also requires further research. It is likely that when feed resources are limited, the acceptance of fungal treated biomass by goats or other ruminants will increase as a result of the need to meet their energy requirements. In addition, as Hai *et al*. (2012, 2013, 2014, 2016) have shown for Siam weed (*Chromonaena odorata*), intake of less palatable feed ingredients may also be increased through *in utero* exposure. As such, increased intake of fungal treated biomass could also be achieved by inclusion of the feed ingredient in the maternal diet, either during late gestation or potentially lactation.

Many organic acids and soluble sugars in mushrooms contribute to their flavour (Chen *et al*., 2015). The organic acids and sugars generated during the fungal treatment might also enhance (or reduce) the palatability of treated substrates for animals. The anaerobic storage of fungal treated wheat straw, especially at higher temperatures, might be a great advantage, because more water soluble sugars are “liberated” which can enhance the flavour (Chapter 4). The organic acids and other compounds also accumulate simultaneously, but this can be either an advantage or disadvantage, since these components might enhance or decrease the flavour, and might affect the rumen microbiota, for example oxalic acid (Duncan *et al*., 1997, 2000). It is thus essential to quantify the produced organic acids and to study the effect of the major acids on the

activity of rumen microbes. This might provide information for feeding strategies of fungal treated wheat straw in practice.

The darker colour of the fungal treated wheat straw stored at higher temperatures was caused by the solid fraction of the material, as repeated washing with water showed very little change in colour (Chapter 4). The darker colour can result from complex interactions between various components, such as the occurrence of the Maillard reaction, caramelisation and changes in phenolic compounds. Although none of these reactions were investigated in Chapter 4, the required components and conditions involved in the reactions exist during storage (e.g. reducing sugars, amino acid, high temperature, etc.) (Chapter 3 and 4; Yang *et al*., 2001). The Maillard reaction and caramelisation are commonly used processes in the production of food (humans and pets) to add colour and flavour. The latter might also be important for the higher acceptance of the stored, compared to the fresh fungal treated substrate by the goats (Chapter 5). Further research is required to determine the compounds which were responsible for the flavour and the colour changes.

7.7 Optimization of colonization by fungi

The optimization of fungal growth is one of the important factors for a successful and efficient conversion of biomass to ruminant feed. White-rot fungi degrade easily accessible water soluble compounds for their growth during the initial colonization phase. This was reflected in the decrease in *in vitro* gas production after one or two weeks after incubation with *C. subvermispora* and *L. edodes*, where after it significantly increased (Chapter 2 and 6). Wheat straw contains some soluble sugars (Shan *et al*., 2008; Tishler *et al*., 2015), but these nutrients are limited. To continue growth and activity, white-rot fungi need to degrade lignin to access the polysaccharides and hence obtain the necessary carbon and energy sources to continue colonization. Wheat bran is an easily accessible industrial by-product, high in protein, starch and other compounds. Adding wheat bran improved the mycelium content, but did not affect the degradation of lignin (Chapter 6). It is possible that using starch depleted wheat bran might show different results, since the wheat bran used in Chapter 6 had a very high starch content (272.2 g kg^{-1} DM). Secondary metabolic events (e.g. lignin degradation) can be affected by limited availability of nutrients (Jeffries *et al*., 1981; Tudzynski *et al*., 2014) and it might be possible that adding wheat bran affects the production of secondary metabolites produced by fungi.

7.8 Large scale application of fungal treated wheat straw

As mentioned before, fungal treated wheat straw has a great potential to be used as a feed ingredient, despite some existing limitations to allow application on a large scale. A major limitation is that the colonization of white-rot fungi on lignocellulosic biomass need to be carried out under sterile conditions to avoid the risk of contamination by endogenous microbiota. In the current thesis, the wheat straw was pre-autoclaved at 121 °C for one hour before inoculation with white-rot fungi (Chapter 2-6). This method can be considered as a mild version of steam explosion of lignocellulose. This autoclave treatment causes swelling of the fibre structures, leading to an increased enzymatic susceptibility (Fu *et al*., 2012). This relatively harsh process might remove some heat labile nutrients. Moreover, the equipment and high energy requirement make autoclaving inappropriate for individual farmers, hence alternative methods are needed. Colleagues during my studies reported here, investigated methods of pre-treating wheat straw with steam or hot water at lower temperatures, showing promising and advantageous results compared to autoclaving. *C. subvermispora* and *L. edodes* growth on wheat straw occurred without contamination during the incubation for several weeks (Ratni *et al*., unpublished observations). However, depending on the pre-treatment length with hot water, soluble sugars released from wheat straw will be removed during the required draining of the water. It is worth noting that the selected method should be in accordance to the characteristics of the biomass. Although using hot water or steam as a replacement for autoclaving shows some advantages, it might only be suitable for soft lignocellulosic biomass, and not for more woody type biomass containing a very high lignin content.

In addition, wheat bran is an ideal substrate for fermentation by heat-producing bacteria. In this way, substrates can be heated to high temperatures (approximately 70 °C) during a natural fermentation process, inactivating most of the bacteria. This method can be a good candidate for the replacement of autoclaving, especially when wheat bran contains a high starch content (Chapter 6). A milder heating process also has less effect on heat labile nutrients and soluble sugars from wheat bran, as they can be used by the fungi for a better colonization.

7.9 Recommendations and future perspectives

7.9.1 The potential methods for supplying a clean environment for fungal incubations

Ensiling of wheat straw before treatment with aerobic white-rot fungi may also have the potential to be used as a pre-treatment to eliminate aerobic microorganism or produce a strong acidic environment to eliminate acid-intolerant microorganism. However, this method has disadvantages since the growth of fungi can be suboptimal when the acidity is outside the range of optimal fungal growth, even though most white-rot fungi prefer slight acidic conditions. Hence, after ensiling, washing might be needed (Thomsen *et al*., 2016). Most of the acids and metabolites produced as a result of ensiling can be eliminated by washing steps, as well as other acids and anaerobic tolerant microorganisms. However, also water soluble sugars and nutrients present will be removed.

7.9.2 More efficient colonization on biomass and its utilization

To achieve a more rapid colonization and more efficient lignin degradation, further work to select and/or breed more effective white-rot fungi is necessary. Next to the selectivity and extent of lignin degradation, also the optimum conditions for growth, such as temperature, moisture, oxygen content and substrate for specific fungal strains are important to explore. To reduce the utilization of spawn, using previously prepared fungal treated wheat straw as an inoculator on new biomass can be an efficient alternative. The advantages of this method is the lower costs and often a reduction in time to colonize the substrate since the fungus does not need time to adapt to a new substrate composition. Small farmers could use a small part of a previously generated fungal treated batch to inoculate a new batch of substrate. It might also be possible to use the rest substrate after the harvest of mushrooms as an animal feedstuff although the nutritional value may be poor due to the use of carbohydrates for the formation of the fruit bodies.

C. subvermispora and *L. edodes* can grow well on cacao hulls, citrus pulp, oat husks, palm kernel meal, rape seed meal and wheat straw pellets (Mao, unpublished observations). It is, therefore, interesting to further analyse nutrient changes to see the potential of these biomasses to be developed into an animal feed ingredient. Considering the difference in water absorbance and chemical composition, the soaking strategy and fungal incubation length might be different for these biomasses compared to wheat straw.

7.9.3 Effect of fungal treatment on animal derived foods

The compounds accumulated in fresh or stored fungal treated substrates might affect the quality of animal products, such as meat and milk. In the present work, the animals used in the preference experiment (Chapter 5) were non-lactating goats. The goats were not slaughtered for meat analysis. It might be important to study the influence of feeding animals with fungal treated wheat straw for a prolonged period on animal performance, including meat and milk quality. Besides ruminant animals, it might be interesting to explore the possibility to feed fungal treated wheat straw to non-ruminant animals such as pigs (e.g. sows).

7.9.4 Reduce the loss of nutrients during storage

When storing fungal treated wheat straw (or other biomass) on a large scale, the high moisture content can results in the accumulation of free water at the bottom of a silo. Solubilized nutrients (e.g. soluble sugars) can be lost in this liquid. Reducing the water content of the fungal treated wheat straw is important to reduce the water loss during storage. However, reducing the soaking time from three days to overnight did not result in a reduced moisture content, as the moisture content of wheat straw after soaking overnight (80.2%, Chapter 5) was similar to soaking for three days (78-82%, Chapter 2, 3, 4 and 6). In the current work, a moderately long draining period of 5 h was used (Chapter 2-6). Further research needs to be conducted to determine if the moisture content can be reduced after soaking. Care needs to be taken to ensure that no undesirable microorganisms will grow during this stage and produce mycotoxins.

7.9.5 Feed-out period

Deterioration of stored fungal treated substrate during the feed-out period is a potential problem, because undesirable microorganisms can regrow, resulting in loss of DM and the potential production of mycotoxins. In addition, white-rot fungi might become active again when exposed to oxygen during the feed-out period. Although it can be seen that *C. subvermispora* and *L. edodes* regrow when the fungal treated wheat straw is continuously exposed (at room temperature) to oxygen (Mao, unpublished observations), other microorganisms may also grow on the surface. It is interesting to investigate the stability of fungal treated wheat straw when exposed to oxygen for a short period of time (< 3 d). Here, the storage temperature should be taken into account because white-rot fungi have

less or no chance to regrow during exposure to oxygen, after the storage at high temperatures, unlike other undesirable air-borne microorganisms.

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Summary

Selective white-rot fungi have great potential to upgrade agricultural by-products into animal feed. Here, the nutritive quality, safety (mycotoxins), storage stability and acceptance by goats of fungal treated wheat straw are reported.

Two white-rot fungi (*Ceriporiopsis subvermispora* and *Lentinula edodes*) were selected and wheat straw was used as substrate. In **Chapter 2**, wheat straw was treated with the two fungi and stored anaerobically 'as is', with the addition of lactic acid bacteria or with the addition of a combination of lactic acid bacteria and molasses. The wheat straw was acidified (pH < 4.3) by the two fungi providing an advantage for the storage of the biomass, as fungal treated wheat straw can successfully be anaerobically stored without the addition of additives. Minor changes in fibre composition and fermentability in rumen fluid were observed after 64 days of storage. For a better understanding of the nutritive quality of fungal treated wheat straw, diverse compounds generated during the fungal treatment were investigated in **Chapter 3**. The principle component analysis of data obtained by gas chromatography and mass spectrometry (GC-MS) showed that the largest changes in compounds profile occurred during four weeks of fungal incubation and differed significantly between the two fungi. The detected compounds were mainly comprised of organic acids and sugars, which accumulated or decreased during the eight weeks of fungal treatment. Ceriporic acid A, B, C and G, specific for *C. subvermispora* were detected in *C. subvermispora* treated wheat straw and showed an increase in the first four weeks. None of the 34 analysed mycotoxins were found to be present in both fungal treated wheat straw after 8 weeks of incubation.

For practical application, it is important that fungal treated wheat straw is a stable product when stored under variable climatic conditions. As such, in **Chapter 4**, *C. subvermispora* and *L. edodes* treated wheat straw were anaerobically stored at different temperatures (24.7, 35.0, 45.9 and 52.4 °C) for 10 weeks. Small but significant changes in hemicellulose and lignin (ADL) content of the fungal treated wheat straw were observed, especially when stored at 52.4 °C. The fungal treated wheat straw became darker in colour at higher temperatures, and an accumulation of some organic acids and sugars were observed when stored under anaerobic conditions. The degradability in rumen fluid was, however, not affected by storage conditions.

To successfully use fungal treated biomass as an animal feed ingredient in practice, the acceptance by animals is important. In **Chapter 5**, three preference studies were conducted with goats. When offered as a single feed, all goats showed the greatest

preference for grass silage, only minor consumption of maize silage, and they avoided to consume fresh fungal treated wheat straw as well as wet wheat straw. Goats showed a significant increase in intake of wheat straw and fungal treated wheat straw when used as an ingredient (50% on fresh basis) in a grass and maize silage based diet. The intake of fungal treated wheat straw was significantly higher than wheat straw in the mixed diet, whereas no significant difference was observed between the two fungal treated wheat straws. Storage of both fungal treated wheat straws resulted in a higher intake by goats than the fresh treated wheat straw.

A fast colonization and sufficient mycelium growth are important to protect wheat straw from contaminations. In **Chapter 6**, wheat bran was added to wheat straw to achieve a faster colonization and more efficient lignin degradation by *C. subvermispora* and *L. edodes*. A quicker colonization and increased mycelium content was observed with an increased concentration of wheat bran added when treated with *C. subvermispora*. The addition of wheat bran also had a positive effect on the fungal biomass of *L. edodes*. The two fungi showed a different pattern of starch degradation, with *C. subvermispora* significantly degrading starch in two weeks whereas *L. edodes* degraded starch in a more linear manner until 12 weeks. The addition of wheat bran had no significant effect on lignin degradation. Also no effect of wheat bran addition on *in vitro* degradation was observed.

This thesis shows that fungal treated wheat straw can be stored anaerobically during long periods of time and that it retains its nutritional value. Fungal treated wheat straw is well accepted as part of a mixed diet by goats and more preferred compared to the untreated straw. Storage of fungal treated wheat straw at elevated temperatures also provides a strategy to increase its palatability. None of the known mycotoxins were detected in the fungal treated wheat straws.

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About the author

Curriculum vitae

List of publications

Training & Supervision Plan

Curriculum vitae

Lei Mao was born on the 10th of August 1988 in Wuzhong, Ningxia, China. In 2011, she obtained her BSc degree in Biochemistry at Sichuan Agricultural University, Ya'an, China. Thereafter, she continued her Master's study in Animal Nutrition and Feed Science at the same University, supervised by Prof. Zhisheng Wang which she obtained in 2014. In October that same year, she started her PhD study at the Animal Nutrition Group in combination with Plant Breeding of Wageningen University & Research, Wageningen, The Netherlands. Her project investigated the storage of fungal treated wheat straw and the acceptance by goats. The results obtained during her PhD period are all presented in this thesis.

List of publications

Peer reviewed scientific publications

Mao, L., Sonnenberg, A.S.M., Hendriks, W.H., Cone, J.W., 2018. Preservation of *Ceriporiopsis subvermispora* and *Lentinula edodes* treated wheat straw under anaerobic conditions. J. Sci. Food Agric. 98, 1232-1239.

Mao, L., Cone, J.W., Hendriks, W.H., Sonnenberg, A.S.M. Wheat bran addition improves *Ceriporiopsis subvermispora* and *Lentinula edodes* growth on wheat straw but not delignification. Submitted to Anim. Feed Sci. Technol.

Mao, L., Marchal, J.L.M., Sonnenberg, A.S.M., Cone, J.W., Hidalgo, V.E., Hendriks, W.H. Preference of fresh and stored *Ceriporiopsis subvermispora* and *Lentinula edodes* treated wheat straw by goats. Submitted to Livest. Sci.

Mao, L., van Arkel, J., Cone, J.W., Hendriks, W.H., de Vos, R.C.H., Sonnenberg, A.S.M. Assessing the nutritional quality of fungal treated wheat straw: Compounds formed after treatment with *Ceriporiopsis subvermispora* and *Lentinula edodes*. To be submitted.

Mao, L., Sonnenberg, A.S.M., van Arkel, J., Cone, J.W., de Vos, R.C.H., Marchal, J.L.M., Hendriks, W.H., The influence of storage temperature and time on feed quality of fungal treated wheat straw. To be submitted.

Conferences and symposia proceedings

Mao, L., Cone, J.W., Sonnenberg, A.S.M., Marchal, J.L.M., Hendriks, W.H., 2017. Conserving wheat straw and fungal treated wheat straw through ensiling. WIAS Science Day, 6 February, Wageningen, The Netherlands.

Mao, L., Sonnenberg, A.S.M., Hendriks, W.H., Cone, J.W., 2017. Potential of fungal treated wheat straw as feedstuff for ruminants. Proceedings 42nd Animal Nutrition Research Forum, 7 April, Ghent, Belgium.

Mao, L., Sonnenberg, A.S.M., Hendriks, W.H., Cone, J.W., 2018. Changes in pH and chemical composition of fungi treated wheat straw, stored anaerobically, with or without additives. Proceedings XVIII International Silage Conference. 24-26 July, Bonn, Germany.

Mao, L., Sonnenberg, A.S.M., Hendriks, W.H., Cone, J.W., 2018. pH of wheat straw during fungal treatment and storage at different temperatures. Proceedings the 10th International Symposium on the Nutrition of Herbivores. 2-6 September, Clermont-Ferrand, France.

Training & Supervision Plan[1]

The Basic Package (3 ECTS[2])

WIAS Introduction Day	2014
Ethics and Philosophy in Life Sciences	2014
Course on essential skills	2014

Disciplinary Competences (12 ECTS)

Writing research proposals	2014
Design of Experiments	2015
Statistics for the Life Sciences	2016
Advances in Feed Evaluation Science	2017
Summer Course Glycosciences	2018

Professional Competences (10 ECTS)

Techniques for Writing and Presenting a Scientific Paper	2015
Project & Time Management	2015
Information Literacy PhD including Endnote Introduction	2015
Data Management Planning	2015
Competence Assessment	2015
Systematic Literature Review	2017
Career Orientation	2018
Writing the General Introduction and Discussion	2018

Presentation Skills (4 ETCS)

WIAS Science Day, Wageningen, Netherlands; poster	2017
Animal Nutrition Forum, Belgium; oral	2017
18th International Silage Conference, Germany; poster	2018
The 10th International Symposium on the Nutrition of Herbivores, France; short poster oral	2018

Teaching Competences (2 ETCS)	
Supervising MSc student	2016
Total ECTS	**31**

[1] Completed in the fulfilment of requirements for the education certificate of the Graduate School, Wageningen Institute of Animal Science (WIAS)

[2] One ECTS credit equals a study load of approximately 28 hours

Colophon

The research reported in this thesis was conducted at the Animal Nutrition Group, Plant Breeding and the laboratory of Bioscience, Wageningen University & Research, Wageningen, The Netherlands. This project was financially supported by the Victam Foundation, Deka Foundation, and ForFarmers through the Wageningen University Fund. The scholarship for the author was provided by the China Scholarship Council. Financial support from Wageningen University & Research for printing this thesis is gratefully acknowledged.

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