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Conversion of Lignocellulosic Biomass Into Valuable Feed for Ruminants Using White Rot Fungi

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ABSTRACT

White rot fungi can degrade lignin and improve the nutritional value of highly lignified biomass for ruminants. We screened for excellent fungi-biomass combinations by investigating the improvement of digestibility of wheat straw, barley straw, oat straw, rapeseed straw, miscanthus, new reed, spent reed from thatched roofs, and cocoa shells after colonisation by *Ceriporiopsis subvermispora* (CS), *Lentinula edodes* (LE), and *Pleurotus eryngii* (PE) (indicated by increased in vitro gas production [IVGP]). First, growth was evaluated for three fungi on all types of biomass, over a period of 17 days in race tubes. CS grew faster than LE and PE on all types of biomass. LE did not grow on cocoa shells, while growth rate of CS and PE on cocoa shells was lower compared to other types of biomass. After this first screening, all types of biomass, excluding the cocoa shells, were colonised by the three fungal strains for 8 weeks. Treatment with CS and LE improved IVGP more than treatment with PE. Methane production was reduced in six combinations of biomass with CS, four with LE, and three with PE. Six types of biomass were selected for treatment with CS and four were selected for treatment with CS and LE, to determine the net improvement of nutritional value (increased IVGP corrected for dry matter loss) after 2, 4, 6, 7 and 8 weeks of treatment. The highest net improvement was found for CS and LE treated rapeseed straw (86% and 20%, respectively) and spent reed (80% and 43%, respectively). All treatments decreased dry matter, lignin and hemicellulose, the latter two both in absolute amount and content. In conclusion, net improvement of highly lignified biomasses by CS was greater than LE, with the nutritional value of rapeseed straw and spent reed being significantly improved by both fungi.

1 | Introduction

Lignocellulosic biomass, a renewable and widely available biological material, is rich in structural carbohydrates (55%–75% dry basis) (Wan and Li 2012; Chio, Sain, and Qin 2019). Various types of biomass are therefore used as animal feed ingredients, particularly for ruminants that have the required microflora to digest those structural carbohydrates. The carbohydrates, that is, cellulose and hemicellulose, form a complex network with lignin, making them less available for the rumen microorganisms (Sufyan et al. 2022; Wu et al. 2023). Lignin is a complex polymeric mixture of three phenolic compounds, that cannot be degraded in the anaerobic environment of the rumen. The presence of lignin has been proven to negatively affect the digestibility of feed by ruminants (Arora and Sharma 2009; van Kuijk et al. 2015a).

Several pretreatments, including physical, chemical, thermal and biological, or a combination of these, have been used to

improve the digestibility of lignocellulosic biomass. Practical limitations for treatments include high costs of equipment, energy, and chemicals, but also cause environmental pollution by some chemicals (e.g., sulfuric acid or sodium hydroxide) (Dashtban, Schraft, and Qin 2009; Sánchez and Montoya 2020). Biological pretreatments using fungi could be an environmentally friendlier alternative for improving the digestibility of lignocellulosic biomasses, which are usually obtained as agricultural and forestry residues (van Kuijk et al. 2015a; Sindhu, Binod, and Pandey 2016). Wood-decaying fungi can roughly be divided into three groups: brown-rot fungi that degrade polysaccharides and modify lignin, soft-rot fungi that simultaneously degrade lignin and polysaccharides, and white rot fungi that selectively degrade lignin with low cellulose loss degradation (Chai et al. 2022). White rot fungi can disrupt the lignocellulose fibre structure by cleaving lignin-polysaccharide bonds, breaking the C-C and ether links of lignin, and by reducing the crystallinity of cellulose. This is done by the combined action of oxidative enzymes and fungal-generated radicals (Dilokpimol et al. 2016; van Erven et al. 2018).

White-rot fungi are considered promising delignifying microorganisms to treat highly lignified biomass and have received increasing attention during the past decades including for applications in ruminant nutrition (Chai et al. 2022). White rot fungi can selectively degrade lignin and make more carbohydrate (cellulose and hemicellulose) available for rumen microbe, thereby improving the nutritional value and digestibility of lignocellulosic biomass as ruminant feed (van Kuijk et al. 2015a; Sufyan et al. 2024). However, the fungi also utilise part of the carbohydrates as carbon and energy source, resulting in overall dry matter (DM) loss during treatment (van Kuijk et al. 2017; Mao et al. 2018; Nayan et al. 2018a; Wang et al. 2023). Therefore, to speak about a net improvement of nutritional value, we need to take into account whether the increased IVGP (as a measure of nutritional value) outweighs the DM loss. Here we came up with the concept of net improvement, and calculated that as the increasing IVGP corrected for DM loss.

Several important factors such as the type of biomass (Tuyen et al. 2012; van Kuijk et al. 2015b; Nayan et al. 2019), fungal species and strain (Tuyen et al. 2012; Nayan et al. 2018a), and cultivation conditions (Wan and Li 2012) determine the success of white-rot fungi in achieving an improvement of the treated biomass in nutritional value. Although some types of biomass like wheat straw (Martens et al. 2022), bagasse, and rice straw have previously been evaluated for their improved nutritional value after fungal treatment (Tuyen et al. 2013; van Kuijk et al. 2015b), the potential for improvement of several other readily available biomasses including barley straw, oat straw, rapeseed straw and reed is, hitherto, unknown.

The present study aimed to determine the net improvement of nutritional value on various types of lignocellulosic biomass for use as ruminant feed (wheat straw, barley straw, oat straw, rapeseed straw, miscanthus, new reed, spent reed, and cocoa shells) after treatment with three white rot fungi: *Ceriporiopsis subvermispora* (CS), *Lentinula edodes* (LE), and *Pleurotus eryngii* (PE). We hypothesise that the lower the nutritional quality of lignocellulosic biomass, as measured by IVGP, the greater the potential for net improvement. Experiments were conducted to determine the growth rate of the three fungal species on these types of biomass. After the changes in in vitro digestibility including methane (CH_4) production of the various types of biomass were determined before and after 8 weeks of treatment with the three fungi. Lastly changes in chemical composition and in vitro digestibility of selected types of biomass after treatment for 0, 2, 4, 6, 7 and 8 weeks with CS and LE were studied.

2 | Materials and Methods

2.1 | Fungal Strains and Spawn Preparation

Fungal strains CS (MES 13094), LE (MES 02121), and PE (MES 13049) are preserved in liquid nitrogen at the Plant Breeding department (Wageningen University & Research, The Netherlands). Strains were cultured on malt extract agar plates in a climate-controlled room at 24°C and 70% humidity for 1−2 weeks until the plates were fully covered by mycelium. For spawn, six pieces of colonised agar (~1.5 cm^2) were placed in a 1.2 L micro box (TP1200+TPD1200; Combiness, Nevele, Belgium) containing ~400 g autoclaved sorghum grains. Sorghum grains were mixed with gypsum ($CaSO_4$) and calcium carbonate ($CaCO_3$) then boiled in water (Recipe: 15 kg sorghum grains, 360 g gypsum, 103 g calcium carbonate, and 20 L of water). Boxes were shaken regularly to disperse the mycelium through the spawn and to prevent clumping of the spawn grains. After incubation for 3−4 weeks, the spawn was fully colonised and stored at 4°C until further use.

2.2 | Biomass Preparation

Wheat straw, and rapeseed straw (Vestjens Straw Products, Haelen), reed and spent reed (Verhoek Riethandel, Genemuiden) and cocoa shells (Gamma, Wageningen), as well as barley straw, oat straw and miscanthus (Unifarm, Wageningen UR) were collected. Spent reed is a by-product generated during the renewal process of thatched roofs, where old, degraded reed material is removed and replaced with new reed to maintain the roof's functionality.

The various types of biomass were chopped to 2−4 cm, except for cocoa shells that were already ~1.5 × 1.5 cm. For race tube experiments, the various types of biomass were ground using a Peppink 100 AN hammer mill (Peppink, Olst, The Netherlands) at 10,000 rpm over a 1 mm sieve. Ground material was transferred into cotton bags that were tightly closed and submerged in tap water for 3 days at room temperature. After soaking, bags were allowed to drain gravimetrically for 3 h, before a final manual pressing removed the remaining excess water. The average final DM content of the various biomasses following the above procedure were (g/kg): wheat straw 205, barley straw 205, oat straw 190, rapeseed straw 215, miscanthus 285, new reed 330, spent reed 370, cocoa shells 295.

2.3 | Biomass Inoculation

2.3.1 | Determining Growth Rate in Race Tubes

A 'race tube' method was conducted to determine the linear growth rate (Straatsma et al. 1989; Zervakis et al. 2001).

Depending on the bulk density of the different types of biomass, between 7 and 11 g (DM basis) of moistened 1 mm particle size biomass was used to fill the race tubes (Ø 22 × L 150 mm) to a height of ~13.7 mm. Biomass was manually compressed using a long iron stick. Tubes were autoclaved at 121°C for 1 h and then left in a laminar airflow cabinet overnight to cool down to room temperature. Two sorghum grains of spawn were added on top of the biomass, under aseptic conditions, before the tubes were closed again with caps and incubated in a climate-controlled room at 24°C and 70% humidity. Mycelium growth was recorded along two lines running from top to bottom of the tube. Twice a week, the front of the mycelium in each tube was measured using a vernier caliper and a stereo microscope and marked on the opposing lines. The experiment was terminated when the mycelium in one of the triplicate tubes reached the bottom (~17 days). All 24 treatments were conducted in triplicate.

2.3.2 | Determining In Vitro Gas Production (IVGP) Changes of Substrates Before and After 8 Weeks of Fungal Colonisation

Depending on the bulk density of the different types of biomass, between 14 and 24 g (DM basis) of moistened biomass was used to fill 0.5 l micro boxes (OV80+OVD80; Combiness, Nevele, Belgium) covered with lids with a #10 white filter (7.44 times of gas exchange per day; Combiness, Nevele, Belgium). Micro boxes were autoclaved at 121°C for 1 h and then left in a laminar airflow cabinet overnight to cool down to room temperature. Spawn was added at a rate of 10% (w/w on a DM basis) to the autoclaved wheat straw and mixed evenly using tweezers under aseptic conditions, followed by incubation at 24°C at 70% relative humidity in a climate-controlled room. Boxes were weighed at Week 0 and after 8 weeks of treatment. All 21 treatments were conducted in triplicate.

2.3.3 | Following Changes in Biomass Composition, IVGP, and Net Improvement During the Fungal Colonisation

Between 26 and 57 g (DM basis) of moistened biomass was used in 1.2 L micro boxes (OV1200+OVD1200; Combiness, Nevele, Belgium), covered with lids with a #10 white filter. Sterilisation, inoculation and incubation were performed as in 2.3.2. After 0, 2, 4, 6, 7 and 8 weeks of fungal colonisation, three boxes of each treatment were stored at −80°C. For chemical analyses and IVGP, samples from −80°C were freeze-dried for 6−7 days, ground to pass a 1 mm sieve using a hammer mill (Peppink, Olst, The Netherlands), and stored at room temperature. All 10 treatments were conducted in triplicate.

2.4 | Sample Analysis

2.4.1 | IVGP and CH_4 Production Measurements

IVGP was determined according to the method of (Cone et al. 1996). The experimental protocol to obtain rumen fluid samples from rumen cannulated cows was conducted under the Dutch Law on Animal Experimentation and approved (approval nr: 2017.W-0042.003) by the Central Authority for Scientific Procedures on Animals (CCD, The Hague, The Netherlands). Rumen fluid was collected from three fistulated Holstein-Frisian cows fed with a maize and grass silage-based diet and mixed with a mineral buffer solution at a ratio of 1:3 under anaerobic conditions by flushing with CO_2. Samples of fungal-treated biomass of 500 mg were added to 60 mL buffered rumen fluid solution and incubated for 72 h in triplicate with the produced gas recorded automatically. The data were corrected for gas produced by the buffered rumen fluid without added sample. At 0, 2, 4, 8, 12, 24, 30, 36, 48, 60 and 72 h after the start of fermentation, 10 μL of gas from the headspace of the bottle was collected by a syringe and injected in a gas chromatograph (Trace 1300, Thermo Fisher Scientific, Waltham, USA) for determination of CH_4 with total CH_4 production during fermentation calculated as described by (Pellikaan et al. 2011).

2.4.2 | Chemical Analyses

The DM content was determined after drying at 103°C for 4 h (ISO 6496), and ash after incinerating for 3 h at 550°C in a muffle furnace (ISO 5984). Neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were determined using an ANKOM fibre analyser 2000 I (ANKOM Technology, Macedon, New York, USA) according to (Van Soest, Robertson, and Lewis 1991). Neutral detergent fibre (aNDFom) was determined with a heat stable amylase, and ADL (sa) was determined by solubilizing cellulose in sulphuric acid. All fibre fractions were expressed exclusive of residual ash. Hemicellulose content was calculated as the difference between NDF and ADF, and cellulose as the difference between ADF and ADL. Crude protein was calculated as N×6.25, with N determined using the Kjeldahl method with $CuSO_4$ as catalyst (ISO 5983).

2.5 | Statistical Analyses

Net improvement of nutritional value after the specified fungal treatment period was calculated as increasing IVGP corrected for dry matter loss:

$$((100\% - \text{DM loss}_{\text{Week } x}) \times \text{IVGP}_{\text{Week } x} - \text{IVGP}_{\text{Week 0}}) / \text{IVGP}_{\text{week 0}} \times 100\%$$

where $x = 2$, 4, 6, 7 and 8 after start of treatment, and DM loss$_{\text{Week } x}$ = dry matter loss (%) after week and x compared to Week 0.

A linear regression analysis with zero intercepts was fitted to the fungal linear growth data of each individual race tube with mycelium front (mm) as the dependent variable and time (days) as the independent variable. The slopes of the regression lines of each individual tube were subjected to a general linear model (GLM) procedure in SAS 9.4 using the following model:

$$Y_{ij} = \mu + B_i + S_j + B_i S_j + e_{ij}$$

where Y_{ij} = response variable; μ = overall mean; B_i = fixed effect of biomass i ($i = 8$; wheat straw, barley straw, oat straw, rapeseed straw, miscanthus, new reed, spent reed, or cocoa shells); S_j = fixed effect of strain j ($j = 3$; CS, LE or PE); $B_i S_j$ =

interaction between biomass i and strain j; e_{ij} = residual error. Methane and the IVGP data of the fungal treatment of the seven biomasses for 8 weeks were subjected to the GLM procedure in SAS using the above model where S_j was replaced by T_j = fixed effect of time j ($j = 2$) and $i = 7$ (wheat straw, barley straw, oat straw, rapeseed straw, miscanthus, new reed or spent reed).

The IVGP and chemical composition data of each biomass data to determine the optimal treatment time were subjected to the GLM procedure in SAS using the following model:

$$Y_i = \mu + T_i + e_i$$

where Y_i = response variable; μ = overall mean; T_i = fixed effect of time respectively. Methane reducti ($i = 6$; 0, 2, 4, 6, 7 or 8 weeks) and e_i = residual error. Differences between treatment means were compared using the means procedure and Tukey's statement for multiple comparisons. The level of statistical significance was declared at $p < 0.05$.

3 | Results

3.1 | Fungal Growth on Different Biomasses

The average linear growth rate of CS, LE and PE on the eight highly lignified biomasses is shown in Table 1. CS and PE grew on all types of biomass, while LE showed no growth on cocoa shells. The linear growth rate of CS on all types of biomass was higher ($p < 0.05$) than that of LE and PE. All fungi showed a linear growth pattern. Of the eight types of biomass, CS grew faster ($p < 0.05$) on rapeseed straw, miscanthus and new reed, and slower on oat straw and cocoa shells. LE grew fastest on oat straw, miscanthus, new reed and spent reed. PE grew fastest ($p < 0.05$) on oat straw, rapeseed straw and miscanthus, and slowest ($p < 0.05$) on cocoa shells.

3.2 | IVGP Changes of Biomasses After 8 Weeks of Fungal Treatment

Table 2 provides an overview of the changes in the IVGP and methane production in comparison to the DM loss of seven types of biomass before (Week 0) and after (Week 8) treatment with the three fungi. Cocoa shells were excluded because of the poor growth rate of the fungi on this biomass. CS improved ($p < 0.05$) the IVGP of all types of biomass in a range of 16% up to 125% with concurrent DM losses of 12% up to 24%. The IVGP values of rapeseed straw, miscanthus, and spent reed increased most, by 125%, 85% and 67% respectively and with DM losses of 19%, 12% and 16%. When treated with LE, wheat straw, rapeseed straw, miscanthus, new reed and spent reed, showed increased ($p < 0.05$) IVGP (19%−54%), with DM losses ranging from 14% to 32%. PE increased ($p < 0.05$) the IVGP of oat straw (18%), rapeseed straw (53%) and spent reed (11%), with accompanying DM losses of 14%, 12% and 8%, respectively.

All fungi reduced ($p < 0.05$) the percentage of CH_4 (1.3% −37.7%) of the total IVGP (Table 2) for almost all types of biomass. The decrease in CH_4 by CS on most types of biomass (12.8%−37.7%) was significant except for wheat straw (10.8%). Methane reduction of LE treated rapeseed straw, miscanthus, new reed and spent reed was 24.6%, 8.8%, 19.6 and 23.5%, respectively. Methane reduction in gas by PE was found for oat straw, rapeseed straw, and spent reed.

3.3 | Net Improvement, IVGP and Chemical Composition Changes Over Time During Treatment

Tables 3 and 4 show the IVGP and DM loss of the different biomasses before (Week 0) and after 2, 4, 6, 7 and 8 weeks of treatment with CS and LE, respectively. PE was excluded because of the low IVGP improvements. Both CS and LE, improved ($p < 0.05$) the IVGP of all selected types of biomass. In

TABLE 1 | Growth rate (mm/day) of *Ceriporiopsis subvermispora*, *Lentinula edodes* and *Pleurotus eryngii* measured over 17 days incubated at 24°C on eight highly lignified biomasses.[a−e]

Biomass	*P. eryngii*	*C. subvermispora*	*L. edodes*
Straw			
Wheat	2.32[c,d]	7.07[b,*]	2.20[b]
Barley	2.54[b]	6.25[c,*]	2.04[b,*]
Oat	2.81[a]	4.15[d,*]	2.44[a,*]
Rapeseed	2.69[a,b]	7.64[a,*]	2.17[b,*]
Miscanthus	2.74[a,b]	7.60[a,*]	2.65[a]
Reed			
New	2.53[b,c]	7.42[a,*]	2.45[a]
Spent	2.30[d]	7.55[a,*]	2.53[a,*]
Cocoa shells	0.88[e]	4.09[e,*]	0
RMSE	0.115	0.146	0.134
p value	< 0.0001	< 0.0001	< 0.0001

Abbreviation: RMSE, root mean square error.
*Values within row differ ($p < 0.05$) from the *P. eryngii* treatment.
[a−e]Values with the same superscript within a column do not differ ($p > 0.05$).

TABLE 2 | In vitro gas production (mL/g organic matter), dry matter loss (%) and percentage (%) of methane of the total in vitro gas production in rumen fluid before (Week 0) and after (Week 8) treatment with *Ceriporiopsis subvermispora*, *Lentinula edodes* or *Pleurotus eryngii* on seven types of highly lignified biomass.[a–e]

		IVGP				% CH_4		
Fungal species	**Biomass**	**Week 0**	**Week 8**	**Change (%)**	**DM loss (%)**	**Week 0**	**Week 8**	**Change (%)**
C. subvermispora	Wheat straw	228[b]	270[b,c,*]	18	24	8.3[e]	7.4[b,c]	11
	Barley straw	266[a]	308[a,*]	16	24	7.8[e]	6.8[c,*]	13
	Oat straw	230[b]	297[a,b,*]	29	24	8.9[d,e]	7.3[b,c,*]	18
	Rapeseed straw	128[d,e]	288[a,b,*]	125	19	11.4[a,b]	7.1[b,c,*]	38
	Miscanthus	104[e]	191[e,*]	85	12	12.6[a]	8.7[a,*]	31
	New reed	179[c]	257[c,d,*]	44	22	9.8[c,d]	7.3[b,c,*]	26
	Spent reed	141[d]	235[d,*]	67	16	10.7[b,c]	7.8[a,b,*]	27
L. edodes	Wheat straw	219[b]	266[a,b,*]	21	32	8.4[c]	7.6[b,c]	10
	Barley straw	251[a]	272[a]	8.0	34	8.3[c]	7.4[c]	11
	Oat straw	223[a,b]	230[b,c]	3.0	41	8.7[c]	8.4[a,b,c]	3.4
	Rapeseed straw	116[d]	173[d,e,*]	49	15	12.2[a]	9.2[a,b,*]	25
	Miscanthus	132[d]	157[e,*]	19	14	10.6[a,b]	9.7[a,*]	8.5
	New reed	172[c]	220[c,*]	26	25	10.2[b]	8.1[b,c,*]	20
	Spent reed	130[d]	200[c,d,*]	54	20	11.5[a,b]	8.8[a,b,c,*]	24
P. eryngii	Wheat straw	237[a]	248[a,b]	5	14	7.8[b]	7.7[b,c]	1.3
	Barley straw	260[a]	287[a]	10	16	7.8[b]	7.4[c]	5.1
	Oat straw	239[a]	283[a,*]	18	14	8.3[b]	7.1[c,*]	15
	Rapeseed straw	139[b,c]	212[b,c,*]	53	12	10.6[a]	7.9[b,c,*]	26
	Miscanthus	124[c]	121[d]	−2.0	6.0	11.2[a]	10.9[a]	2.7
	New reed	162[b,c]	175[c,d]	8.0	10	10.1[a,b]	9.8[a,b]	3.0
	Spent reed	135[c]	149[d,*]	11	8.0	11.3[a]	9.9[a,*]	12

Abbreviations: CH_4, methane; IVGP, in vitro gas production.
[a–e]Values with the same superscript within a column do not differ ($p > 0.05$);
*Values differ ($p < 0.05$) from their respective Week 0 value.

general, the improvement in IVGP of CS treated types of biomass was higher than that of biomass treated with LE. The CS treatment of rapeseed straw (increase of 132 mL/g OM) and spent reed (an increase of 119 mL/g OM) improved ($p < 0.05$) IVGP the most. Dry matter losses following the CS treatment (13%–22%) of biomass were lower than those observed for LE (21%–33%).

The chemical compositions of the biomass before (Week 0) and after 4, 6 and 8 weeks of treatment with CS and LE are shown in Tables 5 and 6. Hemicellulose and ADL content started to significantly decrease ($p < 0.05$) from week 4 in all biomasses and decreased continuously until week 8, except the hemicellulose content of rapeseed straw which remained relatively stable. Both CS and LE degraded the highest amount of ADL on rapeseed straw by 83.7 and 52.6 g/kg, respectively. The lowest losses of hemicellulose caused by CS and LE were also on rapeseed straw, which were 12.7 and 19.7 g/kg, respectively. Both CS and LE increased ($p < 0.05$) the relative cellulose content of most biomasses but there were no changes on miscanthus and rapeseed straw. Both fungi increased ($p < 0.05$) the CP and ash content on the majority of biomasses, except on spent reed. There was also no change in the CP content of CS treated miscanthus.

Figure 1 shows the net IVGP improvements of CS treated biomasses (15%–86%) being higher than that of LE (2%–43%). The highest net improvements of the CS (86% and 80%) and LE (20% and 43%) treatment were on rapeseed straw and spent reed for both fungi. The optimal time points of net improvement for wheat straw and new reed by both fungi were at Week 4, for oat straw, rapeseed straw, and spent reed at Week 7, and for miscanthus at Week 8.

3.4 | Correlation Between IVGP and Fibre Content

Strong linear correlations were found between changes in IVGP and ADL content for CS ($R^2 = 0.96$, $p < 0.05$) grown on six and LE ($R^2 = 0.88$, $p < 0.05$) grown on four types of highly lignified

TABLE 3 | In vitro gas production (mL/g organic matter) and dry matter loss (%) before (Week 0) and after treatment of *Ceriporiopsis subvermispora* on six types of highly lignified biomass.

Week	Wheat straw		Oat straw		Rapeseed straw		Miscanthus		New reed		Spent reed	
	IVGP	DM loss	IVGP	DM loss	IVGP	DM loss	IVGP	DM loss	IVGP	DM loss	IVGP	DM loss
0	210^{b}	—	187^{c}	—	114^{c}	—	97^{c}	—	148^{b}	—	114^{b}	—
2	211^{b}	8^{c}	$196^{b,c}$	4^{d}	110^{c}	5^{c}	76^{c}	3^{d}	138^{b}	2^{d}	130^{b}	5^{d}
4	277^{a}	13^{b}	217^{b}	11^{c}	180^{b}	$9^{b,c}$	136^{b}	5^{d}	213^{a}	10^{c}	204^{a}	$7^{c,d}$
6	270^{a}	21^{a}	284^{a}	$20^{a,b}$	233^{a}	$13^{a,b}$	$154^{a,b}$	8^{c}	214^{a}	15^{b}	223^{a}	$10^{b,c}$
7	284^{a}	20^{a}	298^{a}	21^{a}	246^{a}	$14^{a,b}$	$157^{a,b}$	10^{b}	240^{a}	21^{a}	233^{a}	12^{b}
8	273^{a}	22^{a}	281^{a}	17^{b}	238^{a}	17^{a}	174^{a}	13^{a}	235^{a}	21^{a}	228^{a}	15^{a}
RMSE	19.6	1.1	14.9	1.2	13.6	1.9	19.1	0.9	16.3	0.6	15.6	1.3
p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Abbreviations: DM, dry matter; IVGP, in vitro gas production; RMSE, root mean square error.
[a–c]Values with the same superscript within a column do not differ ($p > 0.05$).

biomass (Figure 2). The regression of CS and LE had slopes of −1.54 and −1.60, respectively.

4 | Discussion

4.1 | Fungal Growth on Different Biomass

Rapid and complete colonisation is essential for a successful fungal treatment of lignocellulosic biomass, as this reduces the chance of infections by competitor microorganisms. It has previously been reported in studies where fungal biomass was estimated through ergosterol, a component of cell membranes specific for fungi and some protozoa, that the colonisation strategy differs between fungal species (Mille-Lindblom, Von Wachenfeldt, and Tranvik 2004; van Kuijk et al. 2015b). CS quickly colonises the biomass primarily during the first week and grows slowly later. In contrast, LE continuously colonises the biomass during the whole treatment period (Tuyen et al. 2012, 2013; van Kuijk et al. 2015a, 2017; Nayan et al. 2018a). Although Martens et al. (2022) utilised ergosterol content as an indicator of fungal growth, there is no clear relationship between ergosterol content and growth rate. In the present study, a race tube method (Straatsma et al. 1989; Zervakis et al. 2001) was used to measure the growth rate of three fungi on eight different types of biomass. Zervakis et al. (2001) suggested that fast mycelial extension is often an indication of a nutritionally poor or unfavourable medium. Indeed, growth rates of CS on untreated types of biomass showing a low IVGP at Week 0 (indicating low nutritional value of untreated biomass; such as miscanthus, rapeseed straw, new reed and spent reed) were higher than on biomass of higher IVGP at Week 0 (wheat, barley, and oat straw). However, growth rates of LE and PE did not follow this rule, and other factors are likely to be involved. In addition, we observed a faster growth rate for CS than for LE and PE on all types of biomass, with the growth rate of LE and PE being comparable. This is probably due to different growth characteristics of different fungal species (Reid 1989). Predictions of growth speed based on the nutritional value of biomass should, therefore, be made with caution.

4.2 | Digestibility Changes of Biomasses After 8 Weeks of Fungal Treatment

IVGP technology can reliably predict in vivo digestibility and nutritional value of feeds for ruminants (Shrivastava et al. 2014; Sharma and Arora 2015). The digestibility of fungal-treated biomass can also be evaluated using the IVGP technique (Cone et al. 1996). In the present study, CS performed overall better than LE, with higher IVGP improvement and less DM loss. PE had the lowest IVGP improvement and DM loss, which is in line with previous studies (Tuyen et al. 2012; van Kuijk et al. 2017). Nayan et al. (2018a) found a significant biological diversity among different fungal strains within species in terms of lignin degradation and IVGP improvement. The fungal strains selected for the present study delignify better and improved the IVGP to a larger extent than other strains used in previous studies (Tuyen et al. 2012; van Kuijk et al. 2015b, 2016; Nayan et al. 2018a) where wheat straw was investigated. We

TABLE 4 | In vitro gas production (mL/g organic matter) and dry matter loss (%) before (Week 0) and after treatment of *Lentinula edodes* on four types of highly lignified biomass.

	Wheat straw		Rapeseed straw		New reed		Spent reed	
Week	**IVGP**	**DM loss**	**IVGP**	**DM loss**	**IVGP**	**DM loss**	**IVGP**	**DM loss**
0	238[b,c]	—	134[d]	—	184[b]	—	141[c]	—
2	215[c]	5[e]	160[c,d]	5[e]	161[b]	4[d]	130[c]	6[c]
4	272[a]	11[d]	172[b,c]	11[d]	230[a]	12[c]	195[b]	7[c]
6	255[a,b,c]	19[c]	195[a,b]	18[c]	232[a]	22[b]	218[a,b]	13[b]
7	280[a]	25[b]	207[a]	22[b]	232[a]	25[b]	235[a]	14[b]
8	270[a,b]	33[a]	194[a,b]	26[a]	223[a]	31[a]	229[a]	21[a]
RMSE	17.6	0.96	16.0	0.55	17.1	1.3	16.7	1.1
p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Abbreviations: DM, dry matter; IVGP, In vitro gas production; RMSE, root mean square error.
[a–d]Values with the same superscript within a column do not differ ($p > 0.05$).

used wheat straw as a control since it was studied extensively in previous research, and we assumed that these strains could also improve the nutritional value of the other selected types of biomass. CS treatment of rapeseed straw, miscanthus, and spent reed, as well as LE treatment of rapeseed straw and spent reed resulted in a relatively greater IVGP improvement than the other fungus-biomass combinations, ranging from 67 to 125 and 49% to 53%, respectively. Rapeseed straw and spent reed thus seem to have a high potential for improving the nutritional value by having it colonised by these fungi. Nasehi et al. (2017) found that treatment of rapeseed straw with *Pleurotus florida* for 3 weeks increased the gas production by 8% and the in vitro OM digestibility 13%. Zadrazil et al. (1996) reported that *P. florida*, *Stropharia rugosoannulata* and *Pleurotus cornucopiae* treatment led to an increase in in vitro OM digestibility of reed stems (15%−33%) and rapeseed straw (28%−48%). While other white rot fungi can also improve the nutritional value of rapeseed straw and reed, the improvements are lower than those resulting of treatment with the fungi used in this study.

The gas produced during in vitro fermentation mainly consists of CO_2 and CH_4, as a result of carbohydrate fermentation in the rumen (Beuvink and Spoelstra 1992; Blu¨mmel and Ørskov 1993). In the present study, CH_4 per unit of total gas produced was reduced in most CS treated biomass (except for wheat straw), in LE treated rapeseed straw, miscanthus, new reed and spent reed, as well as in PE treated oat straw, rapeseed straw and spent reed. These results are consistent with findings from previous studies conducted with different white rot fungi and biomass combinations, which showed that CH_4 per unit of total gas produced was decreased in CS treated oil palm frond (Tuyen et al. 2013), *P. eryngii* treated cassava peels (Barde et al. 2015), as well as LE and PE treated corn straw (Zhao et al. 2020). White rot fungi can degrade lignin, reduce cellulose crystallinity and improve cellulose digestibility (Dilokpimol et al. 2016; van Erven et al. 2018). Methane production and cellulose digestibility were shown to be negatively correlated (Hook, Wright, and McBride 2010). During the fermentation process in rumen fluid, CH_4 is formed by methanogens utilising CO_2 and H_2, produced as a consequence of volatile fatty acids (VFA; acetate, propionate and butyrate) synthesis (Hook, Wright, and McBride 2010). Fungal treatment of biomass changes the fermentation pattern of VFA, increasing propionate synthesis (Nayan et al. 2018a). Moreover, the production of propionate utilizes H_2, resulting in less H_2 being available for CH_4 synthesis and thus less CH_4 production (McAllister and Newbold 2008). The reduction in CH_4 can also be explained by the fact that the fungi produce molecules such as phenols and aldehydes, which reduce the production of CH_4 during anaerobic fermentation (Zhao et al. 2020). Agriculture accounts for 50% of the CH_4 emissions worldwide, with ruminants being the main source (Ellis et al. 2007) and feeding fungal treated biomass rather than the untreated biomass to ruminants could help to reduce CH_4 emissions.

4.3 | Digestibility and Chemical Composition Changes Over Time During Treatment

The delignification of biomass by white rot fungi can be divided into two stages. During the first stage, in the lumen of plant cells, fungal hyphae accumulate and secrete larger molecules in the form of enzymes that cannot penetrate the cell walls with dense structures (Tian, Fang, and Guo 2012; van Kuijk et al. 2015a). The contact of oxidative enzymes with the surface of cell walls stimulates the production of low molecular weight radicals (Ellis et al. 2007) which can penetrate the cell walls to initiate lignin degradation (Kapich, Jensen, and Hammel 1999) and facilitate the diffusion of oxidative enzymes such as lignin peroxidase, manganese peroxidase, versatile peroxidases and laccase (Higuchi 2004). Since there is usually no significant lignin degradation during the first week of treatment, easily accessible components that are not impeded by lignin, such as starch and pectin will be used as the main energy source for fungal growth (van Kuijk et al. 2017). Removal of these components leads to less carbohydrates being available for rumen microbes and, therefore, should result in a decrease in IVGP after treatment with CS and LE during the first 1 or 2 weeks. In the present study, no significant difference in IVGP for all fungal treated biomasses were observed between Week 0 and 2. This is likely caused by the increased carbohydrate availability due to the lignin degradation which replaces starch and pectin as an energy source during Week 2 (van Kuijk et al. 2017).

During the second stage, oxidative enzymes such as lignin peroxidase, manganese peroxidase, versatile peroxidases and laccase

TABLE 5 | Chemical composition (g/kg dry matter) before (Week 0) and after treatment of *Ceriporiopsis subvermispora* on six types of highly lignified biomass.

Biomass	Week	Lignin[e]	Cellulose[f]	Hemicellulose[g]	Crude protein	Ash
Wheat straw	0	70.4[a]	440[c]	262[a]	5.4[c]	26.2[c]
	4	21.6[b]	460[b]	140[b]	5.8[b,c]	31.1[b]
	6	10.3[c]	497[a]	106[c]	6.3[a,b]	33.1[b]
	8	5.3[d]	487[a]	96[d]	6.8[a]	36.3[a]
	RMSE	1.49	6.11	3.9	0.25	1.85
	p value	< 0.0001	< 0.0001	< 0.0001	0.0006	0.0011
Oat straw	0	73.3[a]	425[b]	268[a]	5.8[b]	30.5[b]
	4	44.2[b]	418[b]	198[b]	6.4[b]	34.0[b]
	6	11.4[c]	435[b]	119[c]	7.1[a]	38.5[a]
	8	2.7[c]	454[a]	82[d]	7.4[a]	39.2[a]
	RMSE	4.44	7.33	10.3	0.22	1.53
	p value	< 0.0001	0.0017	< 0.0001	< 0.0001	0.0004
Rapeseed straw	0	117.6[a]	457	134[a,b]	4.9[b]	39.8[a,b]
	4	67.4[b]	446	155[a]	5.7[a]	36.9[b]
	6	39.8[c]	437	140[a,b]	6.4[a]	42.2[a]
	8	33.9[c]	445	121[b]	6.5[a]	42.3[a]
	RMSE	4.99	8.16	9.5	0.30	1.59
	p value	< 0.0001	0.0995	0.0147	0.0006	0.0099
Miscanthus	0	115.2[a]	513	218[a]	2.2	23.0[c]
	4	95.9[b]	521	153[b]	1.9	25.7[b]
	6	85.5[c]	534	156[b]	2.3	27.9[a]
	8	74.9[d]	546	124[b]	2.5	28.6[a]
	RMSE	3.12	16.6	12.1	0.30	0.70
	p value	< 0.0001	0.1611	0.0002	0.2093	< 0.0001
New reed	0	89.0[a]	418[b]	271.5[a]	4.6[c]	41.7[b]
	4	61.8[b]	445[a,b]	164.8[b]	5.0[b,c]	45.5[a,b]
	6	51.8[c]	454[a]	170.0[b]	5.5[a,b]	49.2[a]
	8	42.5[d]	455[a]	149.4[b]	6.3[a]	51.2[a]
	RMSE	2.71	10.9	11.3	0.32	2.67
	p value	< 0.0001	0.0103	< 0.0001	0.0009	0.0204
Spent reed	0	107.1[a]	409[b]	238.8[a]	6.0	45.5
	4	69.7[b]	426[b]	167.8[b]	5.9	46.5
	6	56.9[c]	457[a]	153.2[b,c]	6.0	47.2
	8	49.3[c]	465[a]	135.0[c]	6.1	48.7
	RMSE	3.59	6.93	7.3	0.45	1.66
	p value	< 0.0001	< 0.0001	< 0.0001	0.9703	0.2085

Abbreviation: RMSE, root mean square error.
[a–d]Values with the same superscript within a column per biomass do not differ ($p > 0.05$).
[e]Lignin: acid detergent lignin.
[f]Cellulose: acid detergent fibre—acid detergent lignin.
[g]Hemicellulose: neutral detergent fibre—acid detergent fibre.

play a more important role in lignin degradation than radicals (Higuchi 2004), leading to greater delignification and increased IVGP (van Kuijk et al. 2017; Mao et al. 2021). Both CS and LE significantly improved the IVGP of all selected biomasses, reaching the maximal IVGP value after 4–6 weeks of treatment. In general, CS performed better than LE with higher IVGP improvement and less DM loss. The fibre composition of all treatments changed rapidly during the first 6 weeks with a continuous decrease in ADL and hemicellulose content except for the hemicellulose content of rapeseed straw which remained

TABLE 6 | Chemical composition (g/kg dry matter) before (week 0) and after treatment of *Lentinula edodes* on four types of highly lignified biomass.

Biomass	Week	Lignin[e]	Cellulose[f]	Hemicellulose[g]	Crude protein	Ash
Wheat straw	0	68.1[a]	430[c]	252[a]	5.3[b]	27.9[b]
	4	41.5[b]	467[b]	177[b]	5.8[a,b]	28.8[b]
	6	29.7[c]	498[a]	137[c]	6.4[a]	35.3[a]
	8	27.4[c]	495[a]	127[c]	6.5[a]	36.6[a]
	RMSE	1.86	5.55	4.7	0.29	1.23
	P value	< 0.0001	< 0.0001	< 0.0001	0.0030	< 0.0001
Rapeseed straw	0	117.3[a]	451	155[a,b]	5.2[c]	37.0[b]
	4	85.9[b]	444	189[a]	6.0[b]	41.4[a,b]
	6	72.4[b,c]	436	146[b]	6.5[a,b]	43.9[a]
	8	64.7[c]	428	135[b]	7.1[a]	46.7[a]
	RMSE	5.56	8.87	14.3	0.28	2.31
	P value	< 0.0001	0.0583	0.0090	0.0002	0.0051
New reed	0	88.6[a]	400[b]	286[a]	4.6[b]	44.3[c]
	4	68.6[b]	442[a]	132[b]	4.6[b]	45.6[c]
	6	55.5[c]	460[a]	169[c]	5.9[a]	57.2[b]
	8	48.3[c]	459[a]	150[d]	6.7[a]	64.3[a]
	RMSE	3.83	12.3	6.7	0.40	2.30
	P value	< 0.0001	0.0010	< 0.0001	0.0005	< 0.0001
Spent reed	0	107.5[a]	407[c]	254[a]	5.4	47.8
	4	78.2[b]	429[b]	199[b]	6.1	48.3
	6	67.3[c]	459[a]	170[c]	5.6	50.0
	8	62.4[c]	470[a]	157[d]	6.4	53.0
	RMSE	2.14	8.31	4.3	0.45	2.74
	P value	< 0.0001	< 0.0001	< 0.0001	0.0962	0.1979

Abbreviation: RMSE, root mean square error.
[a–d]Values with the same superscript within a column per biomass do not differ ($p > 0.05$).
[e]Lignin: acid detergent lignin.
[f]Cellulose: acid detergent fiber minus acid detergent lignin.
[g]Hemicellulose: neutral detergent fiber minus acid detergent fiber.

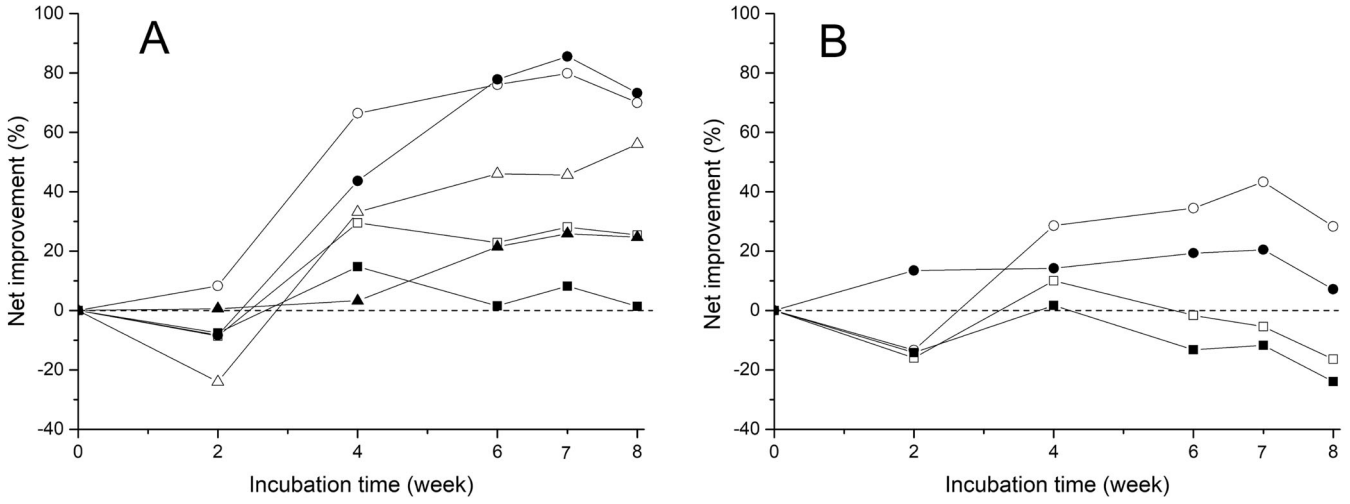

FIGURE 1 | Net improvement of nutritional value of *Ceriporiopsis subvermispora* (panel A) and *Lentinula edodes* (panel B) treatment on wheat straw (■), oat straw (▲), rapeseed straw (●), miscanthus (△), new reed (□) and spent reed (○) over 8 weeks of incubation. (Net improvement was calculated as increasing IVGP corrected for dry matter loss).

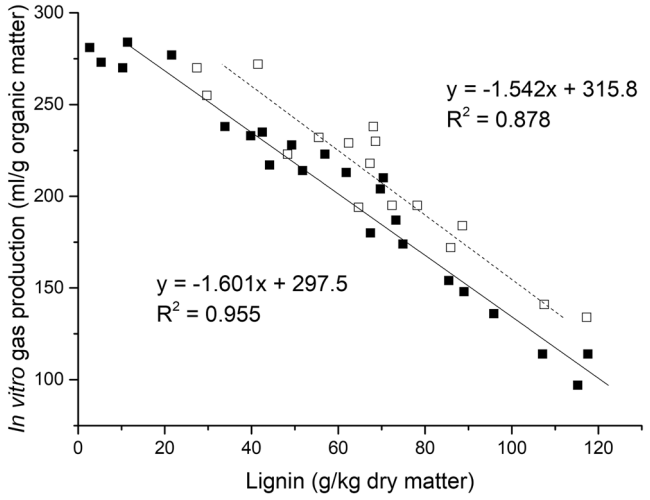

FIGURE 2 | Linear regression of in vitro gas production and acid detergent lignin (ADL) content for *Ceriporiopsis subvermispora* (■) grown on six and *Lentinula edodes* (□) grown on four types of highly lignified biomass for 0, 4, 6 and 8 weeks.

relatively stable. Mainly ADL and hemicellulose were lost in the first 6 weeks, which is consistent with the rapid increase of IVGP in the first 6 weeks. In the present study, the increase of IVGP of fungal treated types of biomass was negatively correlated to the ADL content (Figure 2), which is similar as reported in previous studies (Tuyen et al. 2013; van Kuijk et al. 2015b, 2016). Along with the degradation of ADL, a large portion of the hemicellulose and a small portion of the cellulose is used by the fungi for growth, while the remaining hemicellulose and cellulose are more accessible as an important energy source for the rumen microbes, thus increasing the IVGP. Cellulose content was increased by the CS and LE treatment on all types of biomass, except for the miscanthus and rapeseed straw which remained relatively stable. Expressed as an absolute amount, the cellulose in all treatments was decreased except for the CS treated spent reed which remained stable. Cellulose degradation is limited and cellulose is poorly metabolised by fungi during colonisation (Wan and Li 2012), and the relative increased cellulose content is mainly caused by the removal of hemicellulose. Interestingly, the IVGP of rapeseed straw and spent reed were improved considerably by CS and LE treatment, but the use of carbohydrates from those biomasses by the two fungi seems to differ from that of the other types of biomass. For rapeseed straw, only 24% and 35% of hemicellulose was utilised by CS and LE, considerably lower than for other biomasses (51%−77%). For spent reed, cellulose losses of 4% and 9% were lower than for other types of biomass treated with CS (8%−20%) and LE (20%−30%), respectively. Therefore, there will be more available hemicellulose in rapeseed straw and more cellulose in spent reed after CS and LE treatment, causing a higher IVGP improvement than other biomasses. Increases in CP content result from relative enrichment of nitrogen caused by degradation and respiration with associated DM loss, since white rot fungi are unable to capture nitrogen from air for fungal protein synthesis (van Kuijk et al. 2015a; Nayan et al. 2018b). Fungal treatment with CS or LE can improve the nutritional value of lignocellulosic biomass, but both fungi utilise carbohydrates in the biomass as a carbon source during mycelium growth, resulting in a loss of DM (Tuyen et al. 2013; van Kuijk et al. 2017; Mao et al. 2018; Nayan et al. 2018a).

4.4 | Net Improvement Changes Over Time During Treatment

The effectiveness of a fungal treatment of highly lignified biomass for use in ruminant nutrition depends on net improvement, which is whether the improved nutritional value outweighs the DM loss. Here we came up the concept of net improvement, and calculate as increasing IVGP corrected for DM loss at each treatment time (Figure 1). The highest net improvement of CS treated biomasses (15%−86%) was higher than that of LE (2%−43%). It is important to terminate the treatment at the optimal time, and the time to reach the highest net improvement of both fungi was biomass-dependent. For wheat straw and new reed, this occurred at Week 4, for oat straw, rapeseed straw, and spent reed at Week 7, and miscanthus at Week 8. However, from an application point of view, the highest point of net improvement is not necessarily the optimal time, as equipment and energy costs also need to be taken into account. For most treatments, net improvement increases rapidly in the early stages and at a relatively slow rate in the later stages, indicating that the later stages are not economically efficient. Therefore, a balance needs to be found between improving nutritional value and economic inputs. The optimal time is clear for most treatments, where the highest net improvement and greatest increase appeared at the same time. In contrast, CS treated miscanthus reached the highest net improvement at Week 8, but the greatest increase in improvement at Week 4. For LE, rapeseed straw and spent reed treatments reached the highest net improvement at Week 7, but the greatest increase in improvement at Week 2 and 4, respectively. Since the curve of LE treated rapeseed straw is relatively stable, it only took 2 weeks for LE treated rapeseed straw to reach the 13% net improvement and took 7 weeks to reach the highest net improvement of 20%. Whether the additional 5 weeks of treatment to gain 7% in net improvement outweighs the expense of equipment and energy cost remains to be determined.

Although net improvement is important, there are other advantages to treating highly lignified biomass. Due to the low digestibility of untreated biomass, it will remain longer in the rumen, which reduces the passage rate and thus limits feed intake (Sarnklong et al. 2010). Compared to untreated biomass, fungal-treated biomass becomes more concentrated in nutrients, for example, higher contents of CP and cellulose allowing ruminants to consume more nutrients and feed intake can become less limiting. Mao et al. (2020) found that goats had a higher intake when fed with CS or LE treated wheat straw as compared to untreated wheat straw. Negative correlations were found between the IVGP at Week 0 and net improvement for CS ($r = -0.85$) grown on the six and LE ($r = -0.80$) grown on the four highly lignified biomasses in the present study. The highest net improvements after treatment with CS and LE were found for rapeseed straw (86% and 20%) and spent reed (80% and 43%). The types of biomass with low quality in terms of IVGP have a higher net improvement, indicating that low-quality biomass is more valuable to be converted to ruminant feed. This finding is consistent with findings from previous studies, which showed that the IVGP of low-quality biomasses such as wood chips and oil palm frond were almost doubled after CS or LE treatment (Tuyen et al. 2013; van Kuijk et al. 2015b). Finally, Mao et al. (2021) showed that some organic components are formed

during treatment including various organic acids, carbohydrates, lignin fragments, fungal biomass, and so forth, which may exert bioactivity in the rumen or if isolated, in vivo activity in monogastric animals. Future breeding programs may be able to improve the net improvement value of various fungi making them (more) economically valuable as a treatment of highly lignified biomass for use as animal feed.

5 | Conclusions

Net improvement of highly lignified biomass by *C. subvermispora* was greater than *L. edodes*, with the nutritional value of rapeseed straw and spent reed being significantly improved by both fungi. *C. subvermispora* grew faster than *L. edodes* and *P. eryngii* on eight types of highly lignified biomass. Methane per unit total gas was reduced in most fungus-biomass combinations. In the present study, the types of biomass with low quality (in terms of IVGP) have a higher net improvement, indicating that low-quality biomass has greater economic potential for conversion to ruminant feed.

Author Contributions

Chen Zheng, John Cone, Arend van Peer, Johan Baars, and Wouter Hendriks conceived and designed research. Chen Zheng conducted experiments, analysed data, and wrote the manuscript. John Cone, Arend van Peer, Johan Baars, and Wouter Hendriks supervised the work and corrected the manuscript. All authors read and approved the manuscript.

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Ethics Statement

This article meets EU requirements for the protection of animals and feed legislation. The experimental protocol to obtain rumen fluid samples from rumen cannulated cows was conducted under the Dutch Law on Animal Experimentation and approved (approval nr: 2017.W-0042.003) by the Central Authority for Scientific Procedures on Animals (CCD, The Hague, The Netherlands).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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