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Abstract

Besides their unique ability to depolymerize cell wall components, white-rot fungi are known to assimilate nitrogenous compounds from substrates. This modification may change protein solubility and fermentation in the rumen. To investigate this, the crude protein (CP) in fungal treated wheat straw (3 fungal species, 2 strains each) was fractioned according to the Cornell Net Carbohydrate and Protein System (CNCPS) and assessed for *in vitro* protein fermentation using a modified gas production technique ($IVGP_N$). Results showed that fungi increased fraction A (instantaneously soluble CP; ~2.6 times) and B_1 (rapidly degradable; ~1.2 times); and decreased the slowly degradable fraction B_3 (~41.6%) and unavailable fraction C (~48.3%). The $IVGP_N$ of straw treated with *Ceriporiopsis subvermispora* strains were not different to the control, but increased by 30.2 to 47.1% in *Pleurotus eryngii* and *Lentinula edodes* strains. The $IVGP_N$ was significantly ($P < 0.01$) correlated to all fractions of CP, except fraction B_1 and B_2 (intermediately degradable). All fungi also increased the arginine (~56%) and lysine (~15%) contents. This study shows the importance of assessing the protein solubilization by different fungal strains, which can uncover unique mechanisms in the cell wall depolymerization.

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

1. Introduction

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

Rumen microbes are able to degrade protein from the feed or directly use ammonia and other non-protein nitrogen (NPN) compounds to synthesize microbial N. Utilization of protein from the feed, however, depends on varying proportions of its soluble and insoluble fractions. Different fractions of crude protein in feed have been estimated using the Cornell Net Carbohydrate and Protein System (CNCPS) (Licitra et al., 1996; Sniffen et al., 1992). This method allows partitioning of feed protein into five fractions: Non-protein nitrogen (NPN; fraction A), available true protein (fraction B_1, B_2 and B_3) and unavailable protein (fraction C), which are different in their inherent rates of degradation in the rumen. Fraction A and B_1 are rapidly degraded, while fraction B_2 is fermented at lower rates and can escape the rumen. Fraction B_3 is degraded more slowly due to its association with cell walls and a large proportion escapes the rumen. Fraction C is highly resistant to breakdown in the rumen.

Characterization of the rumen fermentability of the protein fraction in fungal-treated wheat straw has hitherto not been studied. There are a number of *in vitro* techniques to estimate rumen protein fermentation, including enzymatic techniques (Aufrère et al.,

1991; Cone et al., 2004). A modified *in vitro* gas production technique has also been used to estimate protein fermentation characteristics in the rumen (Cone et al., 2009) and large intestine of pigs (Cone et al., 2005). Cone et al. (2009) concluded that there was a good relationship (R^2= 0.85) between the gas production data and the amount of rumen escape protein determined with the nylon bag technique. In brief, the modified technique involves a pre-incubation of diluted rumen fluid with rapidly fermentable carbohydrates in a N-free buffer medium. The approach ensures N to be the limiting factor for microbial growth, so that the subsequent fermentation (and gas production) depend on the availability of N in the samples.

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

2. Materials and Methods

2.1. Preparation of the fungal-treated wheat straw

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

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

2.2. Crude protein fractionation

Crude protein (CP) fractionation was performed according to procedures described by Licitra et al. (1996), with modifications. Samples were analyzed for total N by Kjeldahl analysis (ISO 5983, 2005) and crude protein was calculated as N × 6.25. The non-protein nitrogen (NPN) was obtained by precipitation of true protein in the sample with 10% (w/v) trichloroacetic acid (TCA) for 30 min. After washing 2 times with TCA, the N content in the residues was determined and the NPN was calculated by subtracting residual N from total N. The soluble N was determined by incubating the samples with phosphate-borate buffer (containing 13.79 g/l $NaH_2PO_4 \cdot 2H_2O$ and 8.91 g/l $Na_2B_4O_7 \cdot 10H_2O$ at pH 6.8) at 37°C for 3 h. After incubation, the samples were filtered (Whatman 541, Maidstone, UK) and residual N was determined to yield the insoluble N fraction. The CP contents were presented as absolute amounts, i.e. g per 100 g of starting organic matter (OM). The absolute amount was calculated from the remaining amount (g) of freeze-dried sample, which was corrected for the dry matter content. Neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) were determined by N analysis of the neutral detergent fiber (NDF) and acid detergent fiber (ADF) residues (Van Soest et al., 1991), respectively. All five fractions in Cornell Net Carbohydrate and Protein System (CNCPS) were expressed as a percentage of total CP and were calculated according to Sniffen et al. (1992). These fractions are illustrated in Fig. 1.

2.3. Modified in vitro *gas production*

The modified *in vitro* gas production technique ($IVGP_N$) was performed according to the procedures described by Cone et al. (2009). Rumen fluid was collected from non-lactating cows, fed concentrate and grass silage *ad libitum*. The N-free buffer/mineral solution contained 10.03 g/l $NaHCO_3$, 1.43 g/l Na_2HPO_4, 1.55 g/l KH_2PO_4, 0.15 g/l $MgSO_4 \cdot 7H_2O$, 0.52 g/l Na_2S, 0.017 g/l $CaCl_2 \cdot 2H_2O$, 0.015 g/l $MnCl_2 \cdot 4H_2O$, 0.002 g/l $CoCl_3 \cdot 6H_2O$, 0.012 g/l $FeCl_3 \cdot 6H_2O$ and 0.125 mg/l resazurin. The rumen fluid was diluted 1:19 with buffer/mineral solution to minimize the N input from the rumen fluid. The buffered rumen fluid was pre-incubated with 10 g/l rapidly fermentable carbohydrates, consisting of 3.33 g/l glucose, 3.33 g/l xylose and 3.33 g/l soluble starch, for 5 h at 37°C in a 5 L bottle, under continuous flushing with CO_2. During this pre-incubation, all available N from the rumen fluid was incorporated into bacterial N

components in order to make N limiting for microbial growth. The pre-incubation was also carried out in four 250-ml bottles connected to the gas production equipment for recording the gas production data. After 5 h of pre-incubation, 60 ml of buffered rumen fluid, together with the excess carbohydrates, was added to the bottles with pre-weighed samples containing exactly 5 mg of N (sample DM weight ranged from 1.4 to 2.2 g). Samples were incubated for 72 h and the gas production data were registered automatically. At the end of the incubation, a sample of rumen fluid from each bottle (600 µl) was mixed with 10% trichloroacetic acid (TCA) to determine the ammonia-N (NH_3-N) content spectrophotometrically at 623 nm. The kinetic parameters were determined by fitting the gas production data to a monophasic model (Groot et al., 1996).

2.4. Chemical composition and amino acids determination

Samples were dried for 4 h in an oven at 103°C to determine the dry matter (DM) content (ISO 6496, 1999) with ash content determined after combustion at 550°C for 3 h in a muffle furnace (ISO 5984, 2002). The fiber contents – exclusive of residual ash, were determined using Van Soest et al. (1991), with modification. Neutral detergent fiber (aNDFom) was determined using a heat-stable amylase (thermamyl) and alcalase. Acid detergent fiber (ADFom) and acid detergent lignin (solubilize cellulose with sulphuric acid; lignin-(sa)), were also determined using the standard method. Amino acids were analyzed using the standard AOAC method (AOAC, 2000). The protein in the samples were hydrolyzed with 6 M hydrochloric acids before the individual amino acid composition was separated, detected and quantified using high performance liquid chromatography (HPLC). Tryptophan was not determined. No corrections were made for amino acid loss during hydrolysis, and amino acid weights were calculated using free amino acid molecular weights.

2.5. Statistics

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

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

where Y_{ijk} = response variable *ijk*, μ = overall mean, SP_i = the effect of species *i*, $ST_{j(i)}$ = the effect of strain *j* nested within species *i*, $\tau_{k(i\,j)}$ = effect of week *k*, and ε_{ijk} = residual error with a mean of 0 and variance σ^2. SP_i was considered a fixed effect, $ST_{j(i)}$ and $\tau_{k(i\,j)}$ as random effects. Probability values below 5% were considered significant. Pearson

Product-Moment Correlation (*r*) coefficients were also determined among the measured variables.

3. Results

3.1. Chemical composition and fractionation of crude protein

Table 1 summarizes the chemical composition and the fractionation of CP in wheat straw, treated with different fungal strains. The untreated, autoclaved wheat straw contained 87.2 ± 0.3 g of organic matter (g) per container. All CP fractionation data are presented per 100 g of the starting OM. Fungal pretreatment caused a 1.6 to 7.3% decrease in the total amount of OM by the end of the colonization weeks. The fiber contents were noticeably lower in all fungal-treated wheat straws, particularly the lignin-(sa) content which were decreased by 48.5% in CS1-treated straw. After 7 weeks of colonization, the total amount of CP when expressed per unit starting OM was significantly ($P < 0.001$) increased by 23.3 to 30.9% in the fungal-treated wheat straw, compared to the control. The amount of true protein was lower in straw treated with *P. eryngii* and *L. edodes* strains compared to the control, with the true protein content in PE6-treated straw being significantly ($P = 0.003$) lower. CS1 and CS12-treated straw had a significantly ($P < 0.01$) higher amount of true protein, compared to the control. On the other hand, the amount of NPN was significantly ($P < 0.01$) higher using *P. eryngii* and *L. edodes* strains, compared to *C. subvermispora* strains. All fungal-treated wheat straw had a significantly ($P < 0.001$) higher amount of NPN than the control, except for the CS12-treated straw.

Wheat straw treated with *P. eryngii* and *L. edodes* strains were significantly ($P < 0.01$) lower in the amount of buffer insoluble protein, compared to *C. subvermispora* strains. Contrarily, *P. eryngii* and *L. edodes* strains were higher ($P < 0.01$) in buffer soluble protein than *C. subvermispora* strains. All fungal-treated straw contained a higher ($P < 0.01$) amount of available protein compared to the control. The amount of protein bound to the cell wall was significantly ($P < 0.05$) decreased in fungal-treated wheat straw. The solubilization of the cell wall bound protein resulted in an overall increase of soluble fractions (A and B_1), while the protein fractions related to cell wall (B_3 and C) were decreased by fungal pretreatment.

3.2. In vitro *gas production*

The IVGP during the 5 h pre-incubation with easily fermentable carbohydrates is shown in Fig. 2. A rapid increase of IVGP at a rate of 4.9 ml/ h during the first hour was observed. The rate of IVGP then slowed down to 1.35 ml/h from 2 to 4 h and to 0.9 ml/h

after 4 h. After 5 h of pre-incubation, the buffered rumen fluid was transferred to the gas production unit and the 72 h incubation with the samples started. The uncorrected gas production profiles for all treatments are also shown in Fig. 3. The blanks (N-free buffered rumen fluid with the remaining of fermentable carbohydrates) maintained a gas production lower than 10 ml for the first 18 h of incubation. Nonetheless, the gas production continued in blanks and reached a total IVGP of 34.5 ml after 72 h. Ammonia-N (NH_3-N) was below a detectable amount in all samples of rumen fluid for all treatments after 72 h of incubation.

The corrected gas production profiles ($IVGP_N$) and their kinetic parameters are summarized in Table 2. The $IVGP_N$ of wheat straw treated with *P. eryngii* and *L. edodes* strains was significantly ($P < 0.05$) higher than with *C. subvermispora* strains. Differences in the $IVGP_N$ between wheat straw treated with the *C. subvermispora* strains and the control were not significant. The $IVGP_N$ of CS12-treated straw was even lower than that of the control, although not significant. The kinetics data showed an overall better fermentation profile in all fungal-treated wheat straw as indicated by parameters *B* (half-time of maximum gas production) and R_m (maximum fractional rate of degradation) values.

3.3. Changes in amino acids composition

Glutamic acid (Glu), aspartic acid (Asp), histidine (His), alanine (Ala) and glycine (Gly) were the main amino acids (AAs) present in the untreated (control) wheat straw (Table 3). Overall, there were no marked changes in the total AA–N content when expressed on a CP basis, except for PE3-treated straw which showed a significantly ($P < 0.05$) lower total AA-N content. PE3 significantly ($P < 0.01$) decreased the total essential AAs of the straw by 14.1%. However, all fungi did not cause any significant changes in the non-essential AA content of the straw, although relative decreases were observed in all samples. The changes in the composition of each AA were mostly species-dependent. The strain effect was only significant ($P < 0.05$) for His and methionine (Met). Threonine (Thr), Asp and proline (Pro), however, were not affected by fungal pretreatment. All fungi significantly ($P < 0.05$) increased the arginine (Arg) content of the wheat straw (~56%), but decreased ($P < 0.001$) the Gly content (~19%). Ala was decreased ($P < 0.05$) in wheat straw treated with CS1 (9%), *P. eryngii* (~15%) and *L. edodes* strains (~11%). Glu and Asp contents of the fungal-treated straw were not different to the control.

4. Discussion

The present study encompasses two main parts: (1) fractionation of crude protein content in fungal-treated wheat straw; and (2) *in vitro* assessment of protein availability for the fermentation in the rumen. Results show that fungal colonization leads to an increase in the total amount of CP. An increase in the absolute amount of CP suggests an increase of N in the pre-treated straw. This observation needs a further clarification as it is generally accepted that white-rot fungi are unable to fix atmospheric N and only acquire N bound in biomolecules. In nature, fungi utilize fixed atmospheric N through their symbiotic interactions with nitrogen-fixing bacteria and mycorrhizal fungi (Kneip et al., 2007). Since the straw was sterilized, bacteria cannot play a role in N fixation. The increase in the absolute amount of CP can be attributed to the spawn that was prepared on sorghum grains, which contains ~12% of crude protein (Neucere and Sumrell, 1980). The enrichment of the CP content is also due to the expense of other nutrients (Van Kuijk et al., 2015c). Degraded carbohydrates are converted into fungal biomass and into CO_2 leading to a concentration of N content. Due to the quantitative differences in the absolute amounts of CP (control vs. fungal-treated straw), the fractions are expressed on CP basis to allow comparison among different treatments.

There was no quantitative difference in the total amount of CP among fungal treatments. However, the fractionation of the CP provides a unique insight into the solubilization of protein by different fungal strains. Fungi increased the amount of NPN (fraction A) of the treated straw. The increase of NPN is likely due to the known production of nitrogen-containing secondary metabolites compounds by these higher fungi, such as nitrogen heterocycles, nucleosides, free amino acids (not bound by peptide bonds) and cyclic peptides (Chen and Liu, 2017). Chitin is also an important NPN component of fungal biomass (Ravi Kumar, 1999). The inclusion of chitin N from fungal biomass in fraction A, however, is disputable. Although trichloroacetic (TCA) has been used as chitin solvent (but at a higher temperature or concentration) (Ravi Kumar, 1999), the amount of N from chitin that is solubilized by TCA in this trial was inferred as low. Chitin can also react with α-amino acids to give stable complexes (Tharanathan and Kittur, 2003), which would end up in the true protein fraction. We assume that fraction A in this study does not contain chitin and is directly available for the utilization by the rumen microbes. Therefore, wheat straw treated with CS12, all *P. eryngii* and *L. edodes* strains, had a relatively high content of NPN.

All fungi increased the buffer soluble N fractions (A and B_1) of the wheat straw, especially in *P. eryngii* and *L. edodes* treatments. As expected, there was a decrease in

the cell wall bound proteins (fraction B_3 and C). This observation indicates the capability of fungi to liberate more protein associated with the cell wall. Fraction C is likely solubilized by fungi as a consequence of cell wall polymers degradation. There is indeed a clear correlation of a decrease in fraction C to the amount of lignin ($r = 0.83$; $P < 0.001$) and hemicellulose ($r = 0.76$; $P < 0.001$) – the main components degraded by fungi, but not to cellulose. It is worth noting that, cellulose as calculated by subtracting lignin-(sa) from ADFom using the Van Soest et al. (1991) method, does not exclude chitin (cellulose-like structure with *N*-acetylglucosamine monomers) from fungal biomass. A complete hydrolysis of chitin requires a stronger concentrated acid (Einbu and Vårum, 2008). Protein compounds that are covalently bound to the cell wall, are partly hydrolyzed in the rumen (Debroas and Blanchart, 1993). Thus, fungal pretreatment can contribute to a higher availability of protein for the rumen microbes.

In vitro evaluation of protein fermentation in the rumen provides a complementary finding to the fractionation of protein data. Availability of protein from the gas production was calculated by correcting the values of gas production with blanks. Gas production of the blank is caused by N from the rumen fluid and later on by N released because of microbial turnover (Cone et al., 2009). The incubated samples contained exactly 5 mg N, which made comparison of N availability between the different samples possible. The gas production curves showed a curvilinear to almost linear pattern, indicating a gradual release of N from the samples after the pre-incubation with easily fermentable carbohydrates (Cone et al., 2009). Therefore, a monophasic fit was used, to determine a simple kinetic parameter for the 72 h gas production curve. Results show that only the $IVGP_N$ of straw treated with *C. subvermispora* strains were not different to the control, but shows a better kinetic profile, i.e. a higher fractional rate of the substrate degradation (R_m). The $IVGP_N$ was significantly ($P < 0.01$) correlated to all fractions of CP, except fraction B_1 and B_2. Figure 4 shows the correlation plots of $IVGP_N$ with fraction A, B_1, B_3 and C. We also found significant correlations of $IVGP_N$ to all measured variables, i.e. NPN ($r = 0.78$; $P < 0.001$), buffer soluble protein ($r = 0.68$; $P < 0.001$), NDIP ($r = -0.49$; $P < 0.001$) and ADIP ($r = -0.44$; $P < 0.001$). These observations further support the reliability of using a modified gas production technique in estimating the availability of protein *in vitro* (Cone et al., 2009, 2005). Meanwhile, ammonia-N was below the detection level in the rumen fluid samples, indicating an efficient conversion of available N to microbial N (Cone et al., 1997). This is due to the limited amount of available N in

the buffered rumen fluid, with the only N coming from the samples, which is the limiting factor for microbial growth.

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

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

al. (2015c) did not find a significant improvement in the extent of lignin degradation and *in vitro* degradability with urea supplementation for *C. subvermispora* and *L. edodes* treatments. Removal of N from hemp with protease improved the selective delignification by *Bjerkandera* sp. (Dorado et al., 2001). These reports clearly indicate a large variation among different fungal species in metabolizing protein and affect their selectivity in lignin degradation. In the comparison between CS1 and PE6, CS1 released most of the protein from the cell wall (fraction B_3 and C), but resulted in a lower protein availability (fraction A and B_1). Instead, CS1-treated straw showed a higher buffer insoluble fraction (B_2) compared to PE6. This is probably a mechanism possessed by CS1 to stimulate a higher production of lignin degrading enzymes that may explain its higher selectivity than PE6. Nonetheless, lower protein availability for the fermentation in the rumen is not necessarily a shortcoming as it may also mean that the substrate contains a higher amount of rumen protected protein. This protein can pass through the rumen and may be digested and absorbed in the small intestine of ruminants.

5. Conclusions

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

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Figure Captions

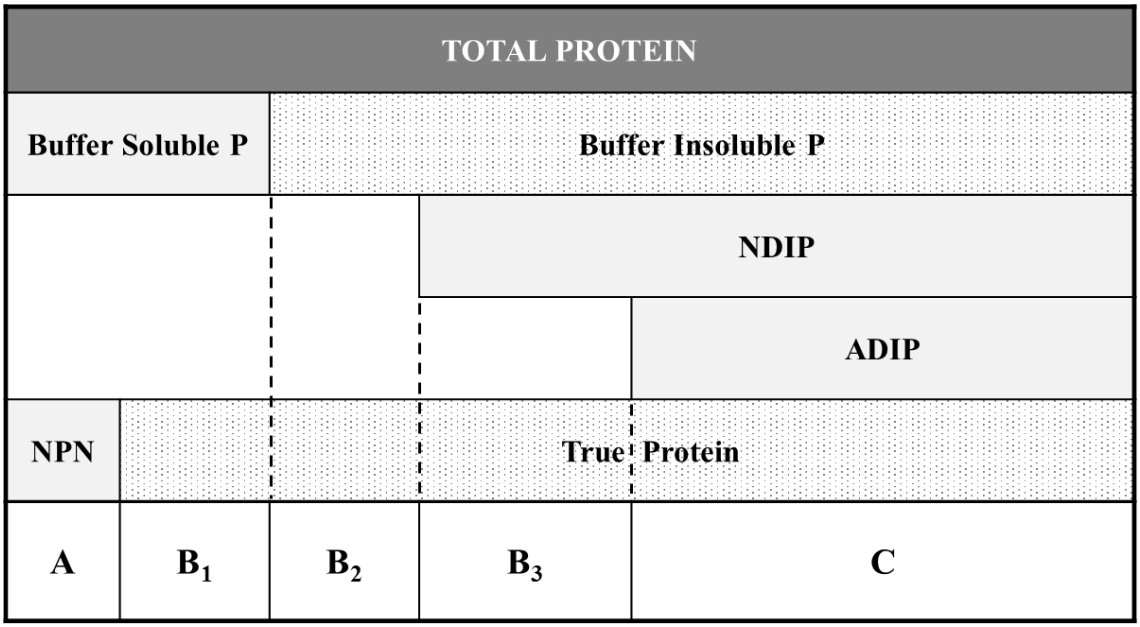


Fig. 1. Fractionation of total crude protein, based on Sniffen et al. (1992). P: protein; NDIP: neutral detergent insoluble protein; ADIP: acid detergent insoluble protein; NPN: non-protein nitrogen.

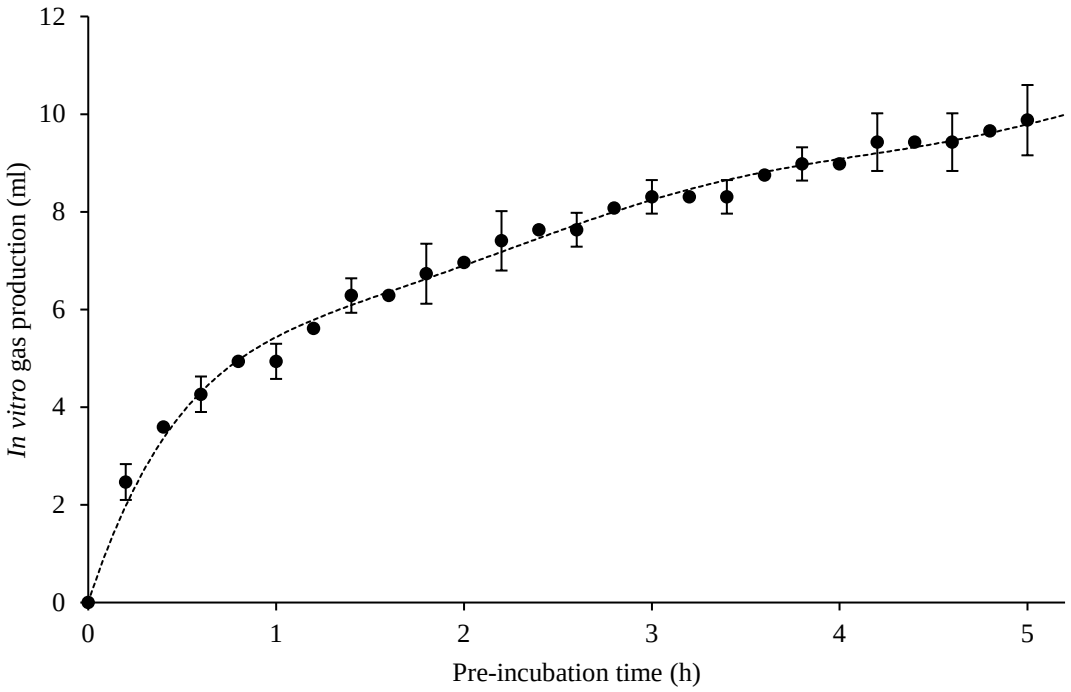
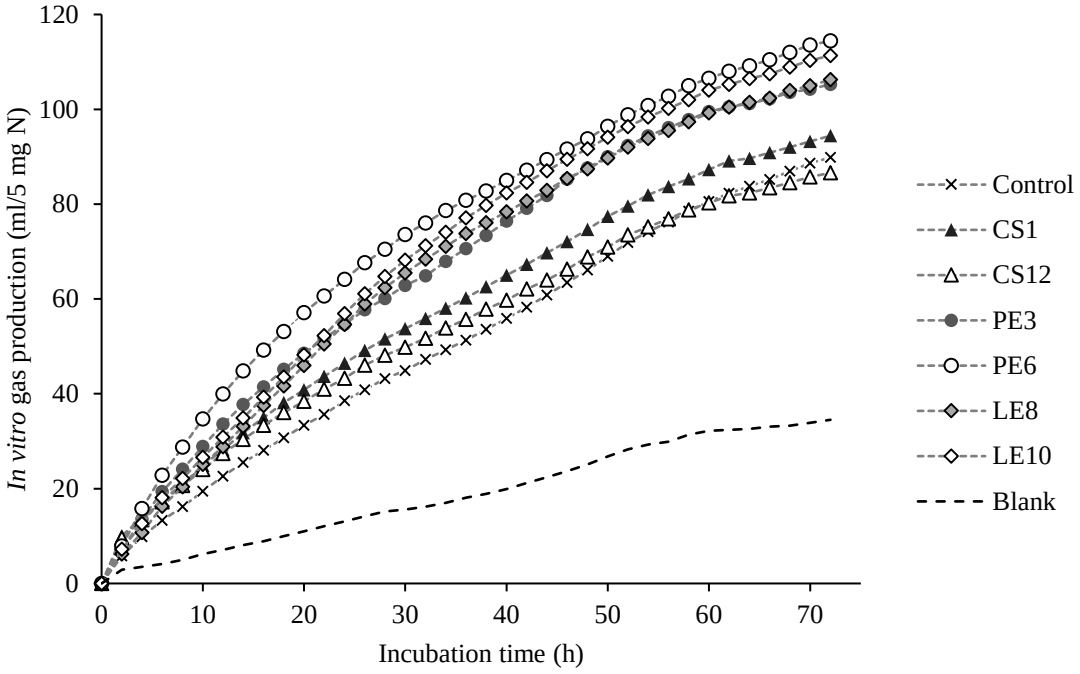


Fig. 2. *In vitro* gas production during pre-incubation with easily fermentable carbohydrates for 5 h. Error bars indicate standard deviation.



471

472 **Fig. 3.** *In vitro* gas production for all fungal-treated wheat straw in comparison to
473 untreated wheat straw (control, ×) and a blank, being buffered rumen fluid without
474 sample (-).The gas production profile were not corrected for the blanks.

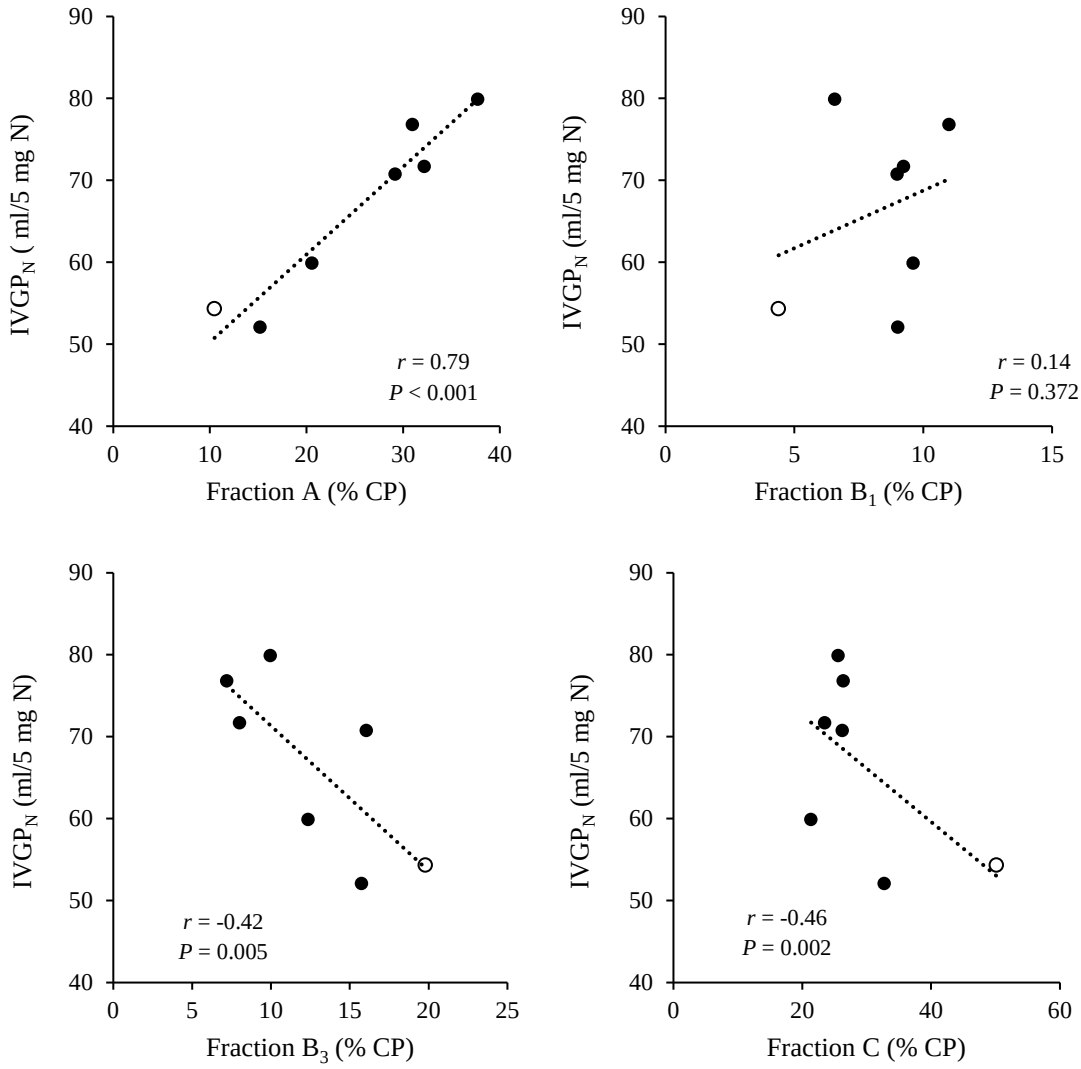


Fig. 4. Correlations among the *in vitro* gas production ($IVGP_N$), soluble protein fractions (A and B_1) and cell wall bound protein fractions (B_3 and C) with respective Pearson's *r* and *P* values. Each point represent mean value for each treatment with 'O' indicates control.

Table 1. Chemical composition and crude protein fractions of wheat straw, treated with different fungal strains for 7 weeks, in comparison to untreated straw (control).

Parameters	Control	CS strain		PE strain		LE strain		RSME	*P* value		
		1	12	3	6	8	10		Sp	St	Time
DM (g/kg)	213.9^{c}	199.9^{a}	209.1^{bc}	213.6^{bc}	209.8^{c}	199.7^{a}	201.8^{a}	2.90	<0.001	0.017	<0.001
Chemical composition (g/kg DM)											
aNDFom	871.7^{f}	701.2^{a}	761.8^{b}	803.9^{d}	828.0^{e}	772.7^{b}	790.0^{c}	6.94	<0.001	<0.001	<0.001
ADFom	585.7^{c}	544.7^{a}	563.6^{b}	571.3^{b}	589.2^{c}	590.8^{c}	587.4^{c}	4.76	<0.001	0.004	<0.001
Lignin-(sa)	83.9^{d}	43.2^{a}	62.3^{c}	63.7^{c}	65.3^{c}	54.6^{b}	60.7^{c}	2.97	<0.001	0.150	<0.001
CP	16.1^{a}	22.3^{b}	20.1^{ab}	21.9^{b}	20.5^{ab}	21.6^{b}	22.5^{b}	2.87	0.034	0.235	0.017
Ash	33.3^{a}	42.5^{e}	40.6^{cd}	41.3^{de}	39.5^{bc}	39.0^{b}	39.5^{bc}	0.80	<0.001	0.052	<0.001
Amount of protein (g/100 g of starting OM) †											
Total CP	1.7^{a}	2.2^{b}	2.1^{b}	2.2^{b}	2.1^{b}	2.1^{b}	2.2^{b}	0.07	<0.001	0.001	<0.001
True protein	1.5^{bc}	1.7^{d}	1.7^{d}	1.5^{c}	1.3^{a}	1.4^{ab}	1.5^{bc}	0.07	<0.001	<0.001	<0.001
Non-protein nitrogen	0.2^{a}	0.4^{b}	0.3^{ab}	0.6^{c}	0.8^{d}	0.7^{cd}	0.7^{cd}	0.08	<0.001	0.124	<0.001
Buffer insoluble protein	1.4^{de}	1.5^{ef}	1.6^{f}	1.4^{cd}	1.2^{a}	1.2^{ab}	1.3^{bc}	0.07	<0.001	0.001	<0.001
Buffer soluble protein	0.2^{a}	0.7^{c}	0.5^{b}	0.8^{d}	0.9^{d}	0.9^{d}	0.9^{d}	0.08	<0.001	0.058	<0.001
NDIP	1.2^{c}	0.7^{a}	1.0^{b}	0.9^{b}	0.7^{a}	0.7^{a}	0.7^{a}	0.06	<0.001	<0.001	<0.001
ADIP	0.8^{d}	0.5^{a}	0.7^{c}	0.6^{bc}	0.5^{ab}	0.5^{ab}	0.6^{bc}	0.07	<0.001	<0.001	<0.001
Protein fractions (% CP) *											
A	10.5^{a}	20.6^{b}	15.2^{ab}	29.2^{c}	37.7^{d}	32.2^{c}	31.0^{c}	3.20	<0.001	0.010	<0.001
B_1	4.4^{a}	9.6^{ab}	9.0^{ab}	9.0^{ab}	6.6^{ab}	9.2^{ab}	11.0^{b}	3.52	0.508	0.037	<0.001
B_2	15.2^{a}	36.1^{d}	27.3^{c}	19.6^{ab}	20.2^{ab}	27.1^{c}	24.5^{bc}	3.16	<0.001	<0.001	<0.001
B_3	19.8^{c}	12.4^{ab}	15.7^{bc}	16.0^{bc}	10.0^{ab}	8.0^{a}	7.2^{a}	3.68	<0.001	0.305	<0.001
C	50.2^{c}	21.4^{a}	32.7^{b}	26.2^{a}	25.6^{a}	23.5^{a}	26.4^{a}	3.26	<0.001	<0.001	<0.001

Values with different superscripts within a row are significantly ($P < 0.05$) different. aNDFom: neutral detergent fiber – exclusive of ash; ADFom: acid detergent fiber – exclusive of ash; CP: crude protein (N × 6.25); NDIP: neutral detergent insoluble protein; ADIP: acid detergent insoluble protein; St: strains; Sp: species.

† Calculated based on the remaining DM (g) after a fungal pretreatment.

* Fractionation of crude protein (as a percentage of CP) based on Sniffen et al. (1992).

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Table 2. Total *in vitro* gas production after 72 h of incubation in buffered rumen fluid and its kinetic parameters for wheat straw treated with different fungal strains for 7 weeks, in comparison to untreated straw (control).

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

Values with different superscripts within row are significantly ($P < 0.05$) different.
$IVGP_N$: Total *in vitro* gas production (modified technique) corrected for blanks; *B*: half time of the maximum gas production (h); *C*: parameters determine curvature of the graph; t_{Rm}: time of the maximum fractional rate of substrate degradation (h); R_m: maximum fractional rate of substrate degradation (h^{-1}); St: strains; Sp: species.
* Fitted to a monophasic curve (Groot et al., 1996).

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Table 3. Content (g/100 g CP) of amino acids (AA) in control (untreated) and fungal-treated wheat straw after 7 weeks of colonization.

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

Values with different superscripts within row are significantly ($P < 0.05$) different. Relative changes of AA contents from control are indicated: More than 50% increase (+++), 20 to 50% increase (++), 0 to 20% increase (+), less than 20% decrease (–) and more than 20% decrease (– –).

* Calculated as the sum of the total moles times number of N atoms in amino acids multiplied by 14.

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