Chemical characterization and in vitro fermentation of *Brassica* straw treated with the aerobic fungus, *Trametes versicolor*

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Ramirez-Bribiesca, J. E., Wang, Y., Jin, L., Canam, T., Town, J. R., Tsang, A., Dumonceaux, T. J. and McAllister, T. A. 2011. **Chemical characterization and in vitro fermentation of *Brassica* straw treated with the aerobic fungus, *Trametes versicolor***. Can. J. Anim. Sci. **91**: xxx–xxx. *Brassica napus* straw (BNS) was either not treated or was treated with two strains of *Trametes versicolor;* 52J (wild type) or m4D (a cellobiose dehydrogenase-deficient mutant) with four treatments: (i) untreated control (C-BNS), (ii) 52J (B-52J), (iii) m4D (B-m4D) or (iv) m4D + glucose (B-m4Dg). Glucose was provided to encourage growth of the mutant strain. All treatments with *T. versicolor* decreased ($P < 0.05$) neutral-detergent fibre and increased ($P < 0.05$) protein and the concentration of lignin degradation products in straw. Ergosterol was highest ($P < 0.05$) in straw treated with B-52J, suggesting it generated the most fungal biomass. Insoluble lignin was reduced ($P < 0.05$) in straw treated with B-52J and B-m4D, but not with B-m4Dg. Mannose and xylose concentration were generally higher ($P < 0.05$) in straw treated with fungi, whereas glucose and galactose were lower as compared with C-BNS. The four treatments above were subsequently assessed in rumen in vitro fermentations, along with BNS treated with 2 mL g^{-1} of 5 N NaOH. Concentrations of total volatile fatty acids after 24 and 48h were lower ($P < 0.05$) in incubations that contained BNS treated with *T. versicolor* as compared with C-BNSor NaOH-treated BNS. Compared with C-BNS, in vitrodry matter disappearance and gas production were increased ($P < 0.05$) by NaOH, but not by treatment with either strain of *T. versicolor*. Although treatment with *T. versicolor* did release more lignin degradation products, it did not appear to provide more degradable carbohydrate to in vitro rumen microbial populations, even when a mutant strain with compromised carbohydrate metabolism was utilized. Production of secondary compounds by the aerobic fungi may inhibit rumen microbial fermentation.

Key words: *Brassica*, *Trametes versicolor*, rumen fermentation, cellobiose dehydrogenase

Ramirez-Bribiesca, J. E., Wang, Y., Jin, L., Canam, T., Town, J. R., Tsang, A., Dumonceaux, T. J. et McAllister, T. A. 2011. **Caractérisation chimique et fermentation in vitro de la paille de *Brassica* conditionnée avec le champignon aérobie *Trametes versicolor***. Can. J. Anim. Sci. **91**: xxx–xxx. Les auteurs ont conditionné ou pas la paille de *Brassica napus* (BNS) avec deux souches de *Trametes versicolor*, 52J (sauvage) et m4D (mutant sans cellobiose déshydrogénase), en fonction de quatre traitements: (i) témoin sans conditionnement (C-BNS), (ii) 52J (B-52J), (iii) m4D (B-m4D) ou (iv) m4D + glucose (B-m4Dg). Du glucose a été ajouté afin de favoriser la croissance de la souche mutante. Tous les traitements avec *T. versicolor* iminuent ($P < 0,05$) la concentration de cellulose au détergent neutre et augmentent ($P < 0,05$) celle de protéines et de produits de dégradation de la lignine dans la paille. L'ergostérol atteint sa concentration la plus élevée ($P < 0,05$) dans la paille B-52J, signe que ce traitement engendre la plus grande masse fongique. La concentration de lignine insoluble diminue ($P < 0,05$) dans la paille B-52J et B-m4D, mais pas dans la paille B-m4Dg. La concentration de mannose et de xylose est habituellement plus élevée ($P < 0,05$) dans la paille conditionnée avec les champignons, mais celle de glucose et de galactose est plus faible que dans chez le témoin. Les quatre traitements ont ensuite été évalués par fermentation in vitro dans le rumen, avec de la BNS traitée avec 2 mL de NaOH 5 N par gramme. Vingt-quatre et 48 heures plus tard, la concentration totale d'acides gras volatils était plus faible ($P < 0,05$) dans les mélanges incubés qui renfermaient de la BNS conditionnée avec *T. versicolor* que dans ceux contenant de la C-BNS ou de la BNS traitée avec du NaOH. Comparativement à la C-BNS, le NaOH accélère ($P < 0,05$) la disparition de la matière sèche in vitroet accroît la production de gaz, ce qui n'est pas le cas pour le traitement avec une ou l'autre souche de *T. versicolor*. Bien qu'il libère plus de produits de la dégradation de la lignine, le traitement avec *T. versicolor* ne semble pas fournir plus d'hydrates de carbone dégradables à la microflore du rumen in vitro, même quand on recourt à une souche mutante dont le métabolisme

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Abbreviations: **BEH**, ethylene bridge hybrid; **BNS**, *Brassica napus* straw; **CDH**, cellobiose dehydrogenase; **DM**, dry matter; **GP**, gas production; **NDF**, neutral-detergent fibre; **RS**, reducing sugars; **VFA**, volatile fatty acids

des hydrates de carbone est déficient. La production de composés secondaires par les champignons aérobies pourrait inhiber la fermentation microbienne dans le rumen.

Mots clés: *Brassica*, *Trametes versicolor*, fermentation dans le rumen, cellobiose déshydrogénase

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Fibrous agricultural by-products as feed for ruminant livestock are plentiful, but their nutritive feeding value is limited by their high fibre, low protein content and reduced digestibility, factors that limit feed intake. Therefore, the majority of these by-products are returned to the land as crop residues shortly after harvest and not used as feed (Graminha et al. 2008). *Brassica* straw is a good example of a crop residue that arises from the harvesting of oilseed crops including *Brassica napus*, *Brassica campestris* and *Brassica juncea*. These species are the three most important *Brassica* species for edible oil production (Hooper 2010) and although almost 6.4 million ha of canola was grown in Canada in 2010 (Canola Council of Canada 2005), almost none of the residue produced was used for livestock feed. While *Brassica* straw contains considerable quantities of cellulose and hemicellulose and has the potential to be a valuable feed source for ruminants, access to these energy-rich carbohydrates by rumen microorganisms is limited by the recalcitrant lignin matrix of straw.

Various methods, including physical, chemical and/or biological treatment (Zadrazil 1977; Sarnklong et al. 2010), have been developed with the aim of increasing the digestibility of straw. Physical methods include grinding, cutting or macerating to facilitate rumen microbial colonization; however, the digestibility of mechanically treated straw is still comparatively low (Varga and Kolver 1997). Previous in vitro and in vivo studies have shown that alkali treatment hydrolyzes ester bonds between hemicellulose and lignin and dramatically improves the ability of rumen microorganisms to ferment straw (Grenet 1997). However, this method is generally expensive, corrosive and can lead to alkalinity in manure-applied soils, factors that have limited its commercial adoption.

Biological treatments of lignocellulosic substrates include cultivation with specific fungi capable of producing a spectrum of lignin and cellulose-degrading enzymes during solid state fermentation. The effects of biological treatment on improving the nutritive value of fibrous forage have been assessed, but conclusions have been variable due to inconsistent treatment outcomes (Permana et al. 2004). The efficacy of a biological treatment depends on the lignolytic activity of the organism. Therefore, it is a prerequisite to identify an organism with high lignolytic activity. As a white-rot fungus, *Trametes versicolor* has the ability to degrade lignin, hemicellulose and cellulose, the principal components of plant cell walls (Valmaseda et al. 1991; Moldes et al. 2004). The enzymes expressed by this basidiomycete include manganese peroxidases (Paice et al. 1993), laccases (phenoloxidases; Bourbonnais et al. 1995) and cellobiose dehydrogenase (CDH; Roy et al. 1996). However, the presence of carbohydrate degrading enzymes in lignolytic fungi can be counterproductive as substrates that could be used by the rumen microbial population are instead utilized during the treatment process. A mutant strain of *T. versicolor* that lacks CDH activity has been previously characterized (Dumonceaux et al. 2001). This mutant is deficient in accessing and utilizing crystalline cellulose as a carbon source, yet retains the lignin-degrading capabilities of the wild-type parent strain. This suggests that it may have the capacity to increase carbohydrate availability through the degradation of lignin, while restricting its utilization of readily degradable carbohydrate.

The objective of this research was to assess the feasibility of using *T. versicolor* (both wild type and a CDH-deficient mutant) as a biological treatment to improve ruminal fermentation of *B. napus* straw as assessed using in vitro techniques.

MATERIALS AND METHODS

Substrate and Treatments

Brassica napus straw (BNS) was obtained from local growers near Saskatoon, Saskatchewan, Canada, and subjected to the following fungal treatments:native BNS ground to pass a 1-mm screen and left (i) untreated (C-BNS) or treated with (ii) sodium hydroxide (B-Na), or *T. versicolor*, (iii) strain 52J, (iv) strain m4D (B-m4D), or (vi) m4D + glucose (B-m4Dg). The B-Nastraw was used as a positive control and was obtained by mixing BNS (40 g) with 20 mL of 5 N NaOH. The straw was allowed to stand at 39°C overnight and was subsequently dried at 50°C to a constant weight.

Cultivation of *T. versicolor* with *B. napus* Straw

Trametes versicolor 52J, a strain that has been investigated as a potential wood pulp biobleaching agent due to its excellent delignification properties (Addleman and Archibald 1993), was obtained from ATCC (96186). *Trametes versicolor* m4D, a mutant strain deficient in the production of cellobiose dehydrogenase (Dumonceaux et al. 2001), was obtained from FP Innovations (Pointe-Claire, QC). An additional treatment, where this strain was supplemented with glucose, was included in order to enhance its growth on BNS.

Prior to fungal treatment, BNS was cut into lengths of 2–4 cm and autoclaved for 30 min at 121°C to kill potential microbial contaminants. Subsamples of autoclaved straw (20 g) were transferred into three 2-L plastic buckets (15 cm diameter × 16 cm height) that had been washed with ethanol and dried prior to use. The straw samples were either left as is (control, C-BNS) or

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inoculated with homogenized *T. versicolor* 52J or m4D that had been grown for 3 d at room temperature in 100 mL of malt extract broth. Each 20 g aliquot of straw was inoculated with 10 mL of each specific blended fungal culture, and the moisture within each solid state fermentation culture was adjusted to 62% by adding water. In the case of B-m4Dg, glucose (15 g L^{-1}) was included in the water used to adjust the moisture level. The buckets were closed with a tight fitting lid and incubated for 12 wk at 23°C. Each bucket was opened weekly in a laminar flow hood to facilitate air exchange.

At the end of 12 wk, samples were dried in a lyophilizer for 3 d, then ground using a Retsch grinder (Newtown, PA) equipped with a 1-mm screen. Dried, ground material was used as input for cell wall composition assays as well as in vitro ruminal fermentation assays.

Chemical Characterization of *B. napus* Straw

Samples were analysed for dry matter (DM) (65°C for 24 h) and neutral-detergent fibre (NDF) using the method of Van Soest et al. (1991) with inclusion of sodium sulphate, but not amylase, in the procedure. Total N was measured by combustion analysis (NA 1500, Carlo Erba Instruments). An acid hydrolysis-based National Renewable Energy Laboratory standard method was used to determine lignin and carbohydrate content of the material (Sluiter et al. 2008). This analysis was not undertaken for the B-Na treatment. After neutralization with $CaCO_3$, 100 μL of the hydrolysate was added to 800 μL of acetonitrile/methanol (75/25; vol/vol) prior to adding 100 μL of 1 mg mL^{-1} fucose (internal standard). After filtration, the individual sugars were detected with an Acquity ultra performance liquid chromatography (UPLC; Waters, Milford, MA), using a 1.7 μm 2.1 × 50 mm ethylene bridge hybrid (BEH) amide column (Waters) with an Acquity tandem quadrupole detector mass spectrometer detector (Waters). A flow rate of 0.25 mL min^{-1} was used with a gradient of 95% acetonitrile/5% isopropanol to 80% acetonitrile/0.1% NH_4OH over 10 min. ESI-ion mode was used with selective ion recording to detect six carbon sugars (m/z 179.2), five carbon sugars (m/z 149.1) and fucose (m/z 163.2). For acid and aldehyde lignin moiety analysis, the neutralized hydrolysate was diluted 10-fold in 50% methanol and filtered prior to separation and detection using an Acquity UPLC (Waters) equipped with a 1.7 μm 2.1 × 50 mm BEH C18 column (Waters) and Acquity photodiode array detector (Waters). A flow rate of 0.25 mL min^{-1} was used with an initial gradient of 95% A (0.1% formic acid)/5% B (acetonitrile) to 65% A/35% B for 4 min, followed by a gradient of 5% A/95% B to 95% A/5% B for an additional 4 min. Acid and aldehyde moieties were detected at 260 and 285 nm, respectively. Total soluble lignin was estimated by absorbance at 240 nm, while insoluble lignin (the filtrate) was determined gravimetrically (Sluiter et al. 2008). Initial biomass values used for lignin determinations were adjusted to account for the biomass contribution from *T. versicolor*. This was achieved by applying the Exponential Growth Stirling Model from SigmaPlot 11 (Systat Software Inc., San Jose, CA) to a data set of experimentally determined ergosterol concentrations against pre-determined *T. versicolor*/*B. napus* mixtures ($P = 0.998$):

$$\text{Fungal biomass (\%)} = \frac{\ln((0.11(([\text{ergosterol}] - 22.2)/7.8)) + 1)}{0.11}$$

Ergosterol was extracted from ground (2 mm) air-dried (room temperature) whole culture using a saponification method as previously described by Hobbie et al. (2009). After extraction, ergosterol in 100% methanol was passed through a 0.2-μm nylon filter before being resolved and detected at 282 nm with an Acquity UPLC (Waters) equipped with a 1.7 μm 2.1 × 50 mm BEH C18 column (Waters) and an Acquity photodiode array detector (Waters). The UPLC flow rate was 0.25 mL min^{-1} using conditions adapted from Lerma-Garcia et al. (2010) whereby a gradient of 20% A (0.1% formic acid)/80% B (acetonitrile) to 5% A/95% B for 30 s followed by 5% A/95% B for 4.5 min.

In vitro Fermentation of *B. napus* Straw in Rumen Fluid

Three batch culture incubations runs were conducted on different days, so that each treatment was conducted in four repetitions at each sampling using the same procedure as described by Wang et al. (2004). Briefly, bottles containing 500 mg of substrate and 40 mL of mixed rumen inoculum were incubated anaerobically at 39°C for 48 h. The inoculum was a 2:1 mixture of mineral buffer (Menke et al. 1979) and rumen fluid. Rumen fluid was collected from two steers feda 75:25 (concentrate: forage) diet, strained through two layers of cheesecloth and combined in equal portions. All animals that served as a source of rumen fluid in this study were cared for according to the guidelines set by the Canadian Council on Animal Care (1993). Gas production (GP) from each bottle was measured using a water displacement device after 12, 24 and 48 h of incubation.

Four vials representing a blank (inoculum only) and each substrate were retrieved from the incubator after 24 and 48 h of incubation. Three vials per treatment were centrifuged at 23 000 × *g* for 10 min. The supernatant was analyzed to determine the concentration of volatile fatty acids (VFA) and ammonia (NH_3) using gas chromatography (5890 Series II; Hewlett Packard, Mississauga, ON). The pellet was processed and analyzed for DM.

Determination of DM Loss and Reducing Sugar Release from *B. napus* Straw

Treated BNS was weighed (100 mg) into 20-mL glass tubes (five tubes per treatment) with two incubations runs on different days, followed by the addition of 5.0 mL of 0.1 Macetate buffer (pH 4.5) and 5.0 mL of a

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mixed rumen enzyme solution with 0.01% (wt/vol) sodium azide, which served as an antimicrobial. The mixed rumen enzyme solution was prepared by collecting 5.0 L of rumen fluid from two steers fed a barley silage and barley grain diet. The rumen fluid was filtered through four layers of cheesecloth and centrifuged at $1000 \times g$ for 10 min to remove feed particles. The resultant supernatant was then sonicated to disrupt bacterial cells using three 30 s pulses separated by 15 s intervals (output 8, duty cycle 60–70%) using a Vibra-Cell processor (Sonics & Materials, Inc., Newtown, CT). The tubes were mixed thoroughly and were capped and incubated with shaking at 39°C. Aliquots (500 μL) of the liquid fraction were taken after 4, 8, 12 and 24 h of incubation and immediately boiled for 10 min to inactivate enzymes. The solution was then analyzed for reducing sugars (RS) using the method described by Wang et al. (2004). After 24 h of incubation, the mixtures were centrifuged at $5000 \times g$ for 20 min and the pellets were dried at 65°C for 48 h to determine DM loss. Three independent incubations were conducted per substrate type with corresponding no-substrate controls serving as blank controls.

Equation and Statistical Analysis

The in vitro dry matter disappearance (IVDMD) from the batch culture incubation was calculated using the following equation:

$$\text{IVDMD} = 1 - [(R - B)/S]$$

Where R is grams of residue of substrate, B is grams of residue of the blank, and S is the dry weight of the initial substrate.

Data were statistically analyzed as a complete randomized design using the MIXED model procedure of the SAS Institute, Inc. (2007) with treatment as the fixed effect. For chemical composition, buckets or individual tube/vial and interaction run × treatment were used as random factor for batch culture and enzyme hydrolysis experiments. The model used for analysis of time-course data (repeated measures) included incubation time and the incubation time × treatment. When interactions with time were significant, treatment means were compared at each incubation time point. Differences among treatments were differentiated using LSMEANS with the PDIFF option.

RESULTS AND DISCUSSION

Growth of Fungal Strains on *B. napus* Straw

Trametes versicolor strains B-52J (wild type) and B-m4D (cellobiose dehydrogenase-deficient mutant) differed widely in their growth on C-BNS during solid-state fermentation. Strain B-52J formed a thick mycelia mass on BNS with visual evidence of penetration of the surface of straw. In contrast, B-m4D grew weakly on BNS, coating the surface of the straw with a thin mycelia layer, but with no obvious penetration. These results were reflected in ergosterol concentrations associated with inoculated BNS as levels were higher ($P < 0.05$) for strain B-52J than B-m4D (Table 1). The addition of glucose to B-m4D-inoculated straw increased its rate of growth on BNS (data not shown), but did not increase the levels of ergosterol produced over the 12 wk incubation period. Despite the weaker growth of B-m4D, there was evidence that all three fungal treatments lowered ($P < 0.05$) the NDF content and increased ($P < 0.05$) the protein content of C-BNS (Table 1).

Concentrations of the lignin degradation products, vanillic acid, syringic acid and 4-hydroxbenzaldehyde, were higher ($P < 0.05$) in BNS incubated with fungi than C-BNS with levels of these compounds generally being greater ($P < 0.05$) for B-52J than B-m4D (Table 1). Release of these products was reflected in a reduction ($P < 0.05$) in the amount of insoluble lignin associated with BNS treated with B-52J and B-m4D as compared with C-BNS (Table 1). Levels of soluble lignin were only higher ($P < 0.05$) with B-52J as compared with other treatments. A reduction in insoluble lignin in BNS treated with *T. versicolor* indicates that both B-52J and the mutant strain B-m4D were capable of delignification. Dumonceaux et al. (2001) also observed that B-m4D was capable of solublizing lignin even though it lacks CDH.

Release of mannose from BNS was increased as a result of treatment with *T. versicolor*, with this increase

Table 1. Concentration of different components, lignin acids and aldehyde moieties released in *Brassica napus* straw treated with *Trametes versicolor*

Treatments[z]	Protein	NDF	Ergos-terol	Vanillic acid	Syringic acid	4-ydroxy-benzal dehyde	Ins-lignin	Sol-lignin	Mannose	Glu-cose	Galac-tose	Xylose	Arabi-nose
	($g\ 100\ g^{-1}$ DM)		($ng\ mg^{-1}$ DM)				($mg\ 100\ mg^{-1}$ DM)						
C-BNS	2.9*d*	81.2*a*	114.0*c*	165.2*c*	98.5*d*	626.7*c*	19.4*a*	1.5*b*	0.9*c*	28.4*a*	2.6*a*	5.2*b*	0.7*a*
B52-J	4.6*b*	68.1*b*	641.1*a*	777.2*a*	561.5*a*	1344.2*a*	14.1*b*	3.5*a*	3.3*b*	18.2*c*	1.5*b*	5.0*b*	0.6*a*
B-m4D	5.2*a*	62.1*d*	244.7*b*	745.6*ab*	489.7*b*	911.3*b*	16.6*b*	2.7*b*	3.2*a*	21.5*b*	1.3*b*	7.0*a*	0.5*a*
B-m4Dg	4.0*c*	65.0*c*	257.5*b*	698.2*b*	366.7*c*	954.5*b*	21.3*a*	2.6*b*	3.5*a*	22.7*b*	1.0*c*	7.1*a*	0.7*a*
SEM	0.9	1.0	31.7	23.8	9.4	91.45	0.8	0.9	0.3	0.7	0.1	0.2	0.1
$P <$	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.01	0.51

[z]C-BNS, untreated native *Brassica napus* straw; B-52J, *Brassica* straw treated with *T. versicolor* 52J; B-m4D, *Brassica* straw treated with *Trametes versicolor* m4D; B-m4Dg, *Brassica* straw treated with *T. versicolor* m4D plus glucose; SEM, standard error mean.
a-d Means in same column with different letters differ ($P < 0.05$).

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being greatest ($P<0.05$) for B-52J. Concentrations of glucose and galactose were lower for BNS treated with *T. versicolor* than C-BNS with arabinose being similar ($P>0.05$) across all treatments. In contrast, xylose was higher ($P<0.05$) in both B-m4D treatments, but B-52J did not differ from BNS. Although still capable of lignin solubilization, it does appear that this mutation impaired carbohydrate utilization as the growth of B-m4D was far lower than wild type B-52J, as reflected by the level of ergosterol production. Despite glucose initially increasing the growth of B-m4D, supplementation with this sugar did not appear to enhance the growth of B-m4D over the 12-wk period. There is a strong positive correlation between the ergosterol content and fungal growth (Lau et al. 2006). Detection of low levels of ergosterol in untreated BNS is likely reflective of the growth of endogenous molds over the incubation period. Dumonceaux et al. (2001) also reported that B-m4D inefficiently utilized crystalline cellulose, despite possessing an intact complement of hydrolytic cellulases. These observations support the hypothesis that CDH acts as an oxidoreductase (Baldrian and Valaskova 2008), and although its biological function remains unknown, it may have a protective function through the reduction of antimicrobial quinones in plants (Morpeth 1991).

Other studies (Asiegbu et al. 1994; Shrivastava et al. 2010) have reported thathydrolytic cellulases from *T. versicolor* released reducing sugars from cellulose and hemicelluloses. Therefore, it is reasonable to predict that treatment of BNS or other lignin-rich substrates with B-m4D straw may render carbohydrates more available, while conserving the cellulose component of the biomass for use by rumen microorganisms.

Previous studies also reported that treatment with white-rot fungi increased the nutritive value of straw (Tripathi et al. 2008; Shrivastava et al. 2010). Wheat straw treated with *Pleurotus ostreatus* and *T. versicolor* had increased CP and decreased organic carbon and C/N ratio as compared with untreated wheat straw. It has been suggested that the decrease in hemicelluloses can be attributed to initial consumption of carbohydrates during mycelial growth (Rodrigues et al. 2008). Jalc et al. (1996) also reported that NDF was reduced when wheat straw was treated with six fungal species. It has been previously shown that white-rot fungi, including *T. versicolor*, can degrade crop residues during solid-state fermentation as a result of action by carbohydrases (cellulases and xylanases) andoxidative ligninolytic enzymes including lignin peroxidases, manganese peroxidase and laccase (Nyanhongo et al. 2002; Singh et al. 2010). The removal of lignin and/or hemicellulose can substantially increase the susceptibility of biomass to enzymatic hydrolysis (Yu et al. 2010), but the enzyme profile produced does not always correlate with the degradation of targeted polymers. This suggests that the extent of fibre degradation is related to more than just the kind or total amount of enzymes produced (Sharma and Arora 2010).

In vitro Fermentation of *B. napus* Straw in Rumen Fluid

Time and time × treatment interactions were observed ($P<0.05$) and therefore data were analyzed and presented at each incubation time point. Gas production from B-Na was higher ($P<0.05$) than that of C-BNS at 12, 24 and 48 h of incubation (Table 2). In contrast, after 12 h, GP of B-m4Dg was similar to that of C-BNS, whereas that of B-52J and B-m4D was lower ($P<0.05$) than C-BNS. After 24 h of incubation, however, GP from all *T. versicolor* treated BNS was lower than that of C-BNS and B-Na. In vitro DM disappearance of B-Na after 48 h of incubation was higher ($P<0.05$) than that of other substrates, which exhibited similar levels of DM disappearance. After 24 h of incubation, NH_3 concentrations of B-m4Dg and B-52J straw were higher ($P<0.05$) than that of C-BNS, B-Na or B-m4D. However, after 48 h of incubation NH_3 concentration of B-Na and B-m4D was higher ($P<0.05$) than C-BNS. Treatment of fibrous residues with NaOH, hydrolyses the ester

Table 2. Effect of different treatments of *Brassica* straw treated with *Trametes versicolor* on the gas production and NH_3 levels during in vitro ruminal fermentation

	IVDMD (g $100g^{-1}$)	Gas production (mL g^{-1} DM)			NH_3 (mM L^{-1})	
Treatments[z]	48h	12h	24h	48h	24h	48h
C-BNS	52.1*b*	27.9*b*	74.2*b*	100.3*b*	0.2*b*	0.3*d*
B-Na	61.1*a*	30.1*a*	89.0*a*	128.0*a*	0.2*b*	0.3*bc*
B-52J	52.0*b*	22.5*c*	59.6*e*	83.9*d*	0.2*a*	0.4*bd*
B-m4D	52.8*b*	23.4*c*	65.4*c*	90.5*c*	0.2*b*	0.4*a*
B-m4Dg	52.7*b*	25.8*b*	71.4*d*	101.6*a*	0.3*a*	0.3*cd*
SEM	0.8	0.8	0.8	1.1	0.0	0.0
P <	0.03	0.01	0.01	0.04	0.01	0.01

Interaction treatment × run: IVDMD: $P=0.9$, GP: $P=0.4$, NH_3: $P=1.0$.

[z]C-BNS, untreated native *Brassica napus* straw; B-Na, *B. napus* straw treated with sodium hydroxide; B-m4D, *Brassica* straw treated with *Trametes versicolor* m4D; B-m4Dg, *Brassica* straw treated with *T. versicolor* m4D plus glucose; B-52J, *Brassica* straw treated with *T. versicolor* 52J; SEM, standard error mean.

a–d Means in same column with different letters differ.

Table 3. Effect of treatments of *Brassica napus* straw treated with *Trametes versicolor* on the volatile fatty acid components during in vitro ruminal fermentation

	Total VFA (mM)		Acetate (mol 100mol^{-1})		Propionate (mol 100mol^{-1})		Butyrate (mol 100mol^{-1})		Acetic:Propionic	
Treatments[z]	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
C-BNS	103.5*a*	150.7*a*	63.4*c*	64.5*bc*	18.5*ab*	18.1*b*	13.5*a*	11.9*ab*	3.5*b*	3.6*a*
B-Na	121.2*a*	130.4*a*	69.0*a*	67.7*a*	17.2*b*	18.5*ab*	10.9*b*	9.8*b*	4.0*a*	3.7*a*
B52-J	50.8*b*	67.6*b*	65.0*b*	64.7*bc*	18.0*b*	18.4*ab*	12.3*ab*	10.8*ab*	3.7*ab*	3.5*a*
B-m4D	69.1*b*	73.2*b*	64.2*ab*	62.7*d*	18.3*ab*	20.7*ac*	13.0*ab*	10.7*b*	3.5*b*	3.1*b*
B-m4Dg	46.8*b*	66.4*b*	64.5*ab*	63.7*bcd*	21.1*a*	20.9*ac*	11.2*ab*	10.2*b*	3.2*b*	3.1*b*
SEM	17.3	14.9	0.6	0.5	1.0	0.9	1.1	1.0	0.1	0.1
P <	0.01	0.01	0.01	0.01	0.04	0.04	0.05	0.01	0.01	0.01

Interaction treatment × run: Total VFA: $P = 0.7$, acetate: $P = 0.1$, propionate: $P = 0.8$, butyrate: $P = 0.9$, A: P: $P = 0.5$.
[z]C-BNS, untreated native *Brassica napus* straw; B-Na, *B. napus* straw treated with sodium hydroxide; B-m4D, *Brassica* straw treated with *Trametes versicolor* m4D; B-m4Dg, *Brassica* straw treated with *T. versicolor* m4D plus glucose; B-52J, *Brassica* straw treated with *T. versicolor* 52J; SEM, standard error mean.
a–d Means in same column with different letters differ.

bonds between hemicellulose and lignin and exposes potentially digestible carbohydrates to microbial attack. Sodium hydroxide treatment of *B. napus*straw has been shown to solubilize phenolic compounds and increases the microbial degradability of dry matter (Alexander et al. 1987), a result that was confirmed in our study.

Concentrations of total VFA were higher ($P < 0.05$) for C-BNS and B-Na than for *T. versicolor* treated BNS after 24 and 48 h of incubation. Concentrations of VFA did not differ among fungal treatments (Table 3). Compared with C-BNS, fermentation of B-m4D produced VFA with lower ($P < 0.05$) molar proportion of acetate, but a higher ($P < 0.05$) molar proportion of propionate after 48h of incubation. Molar proportion of propionate for B-m4Dg was also higher ($P < 0.05$) than that of C-BNS after 48 h of incubation. Furthermore, acetate: propionate ratio of B-m4D- and B-m4Dg was lower ($P < 0.05$) than that of other treatments after 48 h. After 24 h of incubation with a crude mixture of rumen enzymes, all treatments exhibited a similar level of DM loss. In contrast, release of RS was higher ($P < 0.05$) in B-Na after 4, 8 and 24 h of hydrolysis as compared with the control and BNS treated with *T. versicolor*. The degree or reducing sugar release from BNS did not differ among fungal treatments (Table 4).

The similar IVDMD and the reduced gas production and VFA production of fungal-treated BNS compared with C-BNS suggest that the fermentation of BNS was not improved by this treatment. *Trametes versicolor* effectively degrades lignin (Nyanhongo et al. 2002; Singh et al. 2010), as well as other phenolics (Udayasoorian and Prabu 2005) that limit the fermentation of cell wall polymers (Wang et al. 2004). However, the reduced NDF and lignin content of BNS as a result of treatment with *T. versicolor* did not result in an increase thein vitro degradability of BNS. The impact of treatment of straw with white-rot fungi on the degradability of dry matter in crop residues has been variable, from a modest increase (Shrivastava et al. 2010) to no influence or even a decrease (Reid 1989; Jung et al. 1992). This variability likely arises due to differences in the species of white-rot fungi used for treatment, as well as differences in the structural characteristics of the crop residues. It is possible that although treatment with white-rot fungi achieved some delignificaiton, the exposed carbohydrates were preferentially metabolized by the fungi over lignin and more recalcitrant carbohydrates (Tripathi et al. 2008). This scenario would result in the residual material containing less available carbohydrate for fermentation by rumen microorganisms.

In the current study, the decrease in the in vitro concentration of total VFA suggests that treatment of BNS with fungi actually inhibited the fermentative activity of rumen microorganisms. It has been suggested that the fungal nitrogen associated with treated crop residues may be inefficiently utilized by rumen microorganisms (Karunanandaa and Varga 1996), a factor that may have limited, but unlikely inhibited, rumen microbial activity. Rather it seems more likely that

Table 4. Effect of *Brassica* straw treated with *T. versicolor* on dry matter disappearance and reducing sugars release

	Hydrolytic DM loss (g 100g^{-1})	Reducing sugar (μg mL^{-1})			
Treatments[z]	24h	4h	8h	12h	24h
C-BNS	44.6*a*	337.6*b*	371.6*b*	569.5*a*	638.8*b*
B-Na	44.2*a*	452.4*a*	477.1*a*	633.6*a*	831.7*a*
B-52J	45.3*a*	384.5*ab*	240.3*d*	386.5*b*	402.5*c*
B-m4D	47.5*a*	361.0*b*	290.8*bcd*	405.2*b*	393.3*c*
B-m4Dg	45.6*a*	353.6*b*	274.9*cd*	381.1*b*	407.5*c*
SEM	1.4	32.4	32.4	32.4	32.4
P <	0.85	0.01	0.01	0.01	0.01

Interaction treatment × run: HDM loss: $P = 0.2$, RS: $P = 0.2$.
[z]C-BNS, untreated native *Brassica napus* straw; B-Na, *B. napus* straw treated with sodium hydroxide; B-m4D, *Brassica* straw treated with *Trametes versicolor* m4D; B-m4Dg, *Brassica* straw treated with *T. versicolor* m4D plus glucose; B-52J, *Brassica* straw treated with *T. versicolor* 52J; SEM, standard error mean.
a–d Means in same column with different letters differ.

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cellular end products produced by *T. versicolor* may have inhibited the in vitro activity of rumen microorganisms. For example, ergosterol, the principal sterol of fungal cell membranes (Mejanelle et al. 2001; Mattila et al. 2002), has been shown to be easily transformed to its peroxide derivative, which alters the permeability of microbial membranes (Debieu et al. 1992; Veen and Lang 2005; Joffrion and Cushion 2010). Production of other undefined secondary metabolites may also have negatively affected the activity of rumen microorganisms during fermentation.

Treatment with NaOH increased the release of reducing sugars from BNS over the 24 h that it was exposed to a crude mixture of rumen enzymes. This result corresponds with the increased rumen in vitro disappearance of BNS dry matter as a result of NaOH treatment. After 24 h, the release of reducing sugars was noticeably lower for fungal-treated as compared with control BNS. This suggests that the fungal activity did not alter the structure of BNS in a manner that made it more amendable for mixed ruminal enzymes to release reducing sugars from structural carbohydrates. This response is also reflected in the reduced VFA concentrations observed for fungal-treated BNS in in vitro rumen batch cultures.

In conclusion, the treatment of BNS with wild type or CDH-deficient strains of *T. versicolor* solubilised lignin and reduced NDF levels in BNS. These results confirm that CDH is unlikely to play a role in degradation of lignin by *T. versicolor*. However, despite the fact that the mutant strain degraded lignin to a similar extent as the wild type strain, its impaired carbohydrate utilization did not enhance the ability of rumen microorganisms to ferment B-m4D-treated BNS. It appears that secondary metabolites of *T. versicolor* may inhibit the activity of rumen microorganisms and offset any benefit to carbohydrate utilization that may be conferred as a result of the decomposition of lignin. Removal of these secondary metabolites prior to feeding of fungal-treated crop residues may be a prerequisite for this biological treatment to improve ruminal fermentation.

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