Journal of Animal Science, 2020, Vol. 98, No. 5, 1–19

doi:10.1093/jas/skaa116
Advance Access publication May 5, 2020
Received: 5 December 2019 and **Accepted:** April 28 2020
Ruminant Nutrition

RUMINANT NUTRITION

Effect of ammonia fiber expansion-treated wheat straw and a recombinant fibrolytic enzyme on rumen microbiota and fermentation parameters, total tract digestibility, and performance of lambs

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Abstract

The objective of this study was to evaluate the effect of ammonia fiber expansion (**AFEX**)-treated wheat straw pellets and a recombinant fibrolytic enzyme on the rumen microbiome, rumen fermentation parameters, total tract diet digestibility, and performance of lambs. Eight rumen cannulated wethers and 60 lambs (n = 15 per diet, 8 rams and 7 ewes) were used in a replicated 4 × 4 Latin square design digestibility study and a complete randomized growth performance study, respectively. Four treatment diets were arranged in a 2 × 2 factorial structure with AFEX wheat straw (0% or 30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets) and fibrolytic enzyme (with or without XYL10C, a β-1,4-xylanase, from *Aspergillus niger*) as main factors. Enzyme was applied at 100 mg/kg of diet DM, 22 h before feeding. Rumen bacteria diversity Pielou evenness decreased (P = 0.05) with AFEX compared with the control diet and increased (P < 0.01) with enzyme. Enzyme increased ($P \leq 0.02$) the relative abundancies of Prevotellaceae UCG-004, Christensenellaceae R-7 group, *Saccharofermentans*, and uncultured Kiritimatiellaeota. Total protozoa counts were greater ($P \leq 0.04$) in the rumen of lambs fed AFEX compared with control, with enzyme reducing ($P \leq 0.05$) protozoa counts for both diets. Digestibility of DM did not differ ($P > 0.10$) among diets, but digestibility of CP was reduced (P = 0.001), and digestibility of NDF and ADF increased ($P < 0.05$) as AFEX replaced alfalfa. Compared with control, AFEX promoted greater DMI (P = 0.003) and improved ADG up to 42 d on feed (P = 0.03), but not (P = 0.51) over the full ~94-d experiment. Consequently, overall G:F was reduced (P = 0.04) for AFEX when compared with control (0.188 vs. 0.199), but days on feed were lower (P = 0.04) for AFEX (97 vs. 91 d). Enzyme improved DMI of AFEX up to day 70 (P = 0.01), but did not affect DMI of the control diet. Enzyme addition improved ADG of lambs fed both diets in the first 28 d (P = 0.02), but not over the entire feeding period ($P \geq 10$). As a result, G:F was improved with enzyme for the first 28 d (P = 0.04), but not overall (P = 0.45). This study shows that AFEX-treated wheat straw can replace alfalfa hay with no loss in lamb growth performance. Additionally, the enzyme XYL10C altered the rumen microbiome and improved G:F in the first month of the feeding.

Key words: AFEX, ammoniation, lamb, rumen, xylanase, wheat straw

Abbreviations

AFEX	ammonia fiber expansion
AUC	area under the curve
DDGS	Dried distillers' grain with solubles
GC	gas chromatography
MS	mass spectrometry
NFC	non-fiber carbohydrate
YG	yield grades

Introduction

Technologies that can effectively increase fiber digestion have the potential to increase productivity and profitability, and reduce manure production in ruminants, while increasing the use of fibrous feeds unsuitable for humans (Adesogan et al., 2019). Combined, these benefits increase the overall sustainability of ruminant production systems (Adesogan et al., 2019). Cereal straw, a byproduct of grain production, is an abundant low-cost roughage source for ruminants that is not consumed by humans or nonruminants. The low digestibility (<50%) of cereal straw constrains intake and performance, limiting its inclusion in ruminant diets. Development of technologies to enhance the conversion of recalcitrant fiber into energy within the rumen could go a long way to increasing the utilization of crop residues by ruminants.

Ammonia fiber expansion (**AFEX**) is an alkali pretreatment where recalcitrant fiber sources are subjected to steam and anhydrous ammonia at high pressure and temperature for <1 h, followed by a rapid depressurization and ammonia recovery (Mor et al., 2018). Traditional ammoniation methods result in a large portion of the ammonia being volatilized, posing health and safety hazards (Rasby et al., 1989). In contrast, AFEX treatment occurs in reactors where most of the ammonia is recovered and can be reused. Treatment of recalcitrant fiber sources with AFEX disrupts hemicellulose-lignin bonds and cellulose crystallinity, while increasing substrate surface area and solubilizing carbohydrates (Balan et al., 2009). This disruption increases microbial attachment and enzyme access, enhancing the hydrolysis of plant cell walls (Balan et al., 2009). In an artificial rumen (RUSITEC), AFEX treatment of barley straw improved DM disappearance by 35% when compared with untreated straw (62.4% vs. 46.2%; Griffith et al., 2016). Recently, Blümmel et al. (2018) reported that AFEX treatment of various crop residues increased apparent in vitro OM digestibility by 28% (49.3% vs. 63.0%) after 24 h of incubation. The increases in digestibility reported with AFEX cereal straws are approximately 2 times greater than that reported for traditional ammoniation of cereal straws (~15%; Fahey et al., 1993).

Pretreatment of ruminant diets with fibrolytic enzymes can also increase fiber digestibility, growth, milk yield, and feed efficiency as outlined in recent meta-analyses (Arriola et al., 2017; Tirado-González et al., 2018), but responses in several studies have been inconsistent (Adesogan et al., 2014, 2019; Meale et al., 2014). Enzymes not specifically formulated for optimum activity in the rumen and inaccessibility of enzymes to targeted substrates may contribute to this inconsistency (Beauchemin et al., 2003; Adesogan et al., 2014; Meale et al., 2014). Combining ammonia pretreatment of straw with exogenous fibrolytic enzymes increased the effectiveness of enzymes in vitro (Eun et al., 2006) and in vivo (Wang et al., 2004) when compared with the direct application of enzymes onto untreated straw.

The objective of this study was to evaluate the in vivo effects of AFEX-treated wheat straw and the application of a recombinant fibrolytic enzyme (XYL10C) selected specifically to enhance ruminal fiber digestion (Ribeiro et al., 2018). We hypothesized that AFEX wheat straw pellets could replace alfalfa pellets in the diet of lambs without any losses in digestibility and performance. We also hypothesized that this selected recombinant enzyme would result in a greater improvement in fiber degradation with AFEX than alfalfa diets.

Materials and Methods

All lambs were cared for in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009), protocol #1723 approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee. The LeRDC Feed Additive Assessment Committee and the Canadian Food Inspection Agency (CFIA) approved (research authorization no.: 9979532) feeding the enzyme and AFEX-treated straw pellets to lambs destined for human consumption.

Nutrient digestibility and metabolism study

Animals, experimental design, and diets

A total of 8 Canadian Arcott × Rideau Arcott rumen fistulated wethers were used in a nutrient digestibility and metabolism experiment. Wethers (BW; 47.4 ± 3.04 kg) were blocked by weight and randomly assigned to a replicated 4 × 4 Latin square design for measurement of rumen microbiota, fermentation parameters, and diet digestibility. Weathers were housed in individual pens (0.97 × 2.82 m) within a closed barn, and fed 1 of 4 pelleted diets containing 50:50 (DM basis) mixture of forage to concentrate (Table 1). The 4 diets were arranged in a 2 × 2 factorial structure with AFEX wheat straw [0% or 30% AFEX wheat straw pellets (diet DM basis) replacing alfalfa hay pellets] and addition of a fibrolytic enzyme (with or without) as main factors. The recombinant fibrolytic enzyme (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10, from *Aspergillus niger*) was applied at 100 mg of protein per kg of pelleted diet (DM basis) 22 h before feeding. Enzyme was applied the day before feeding to the diet to ensure it had enough time to act on the substrate before animals were fed. The appropriate enzyme concentration was diluted in water and 15 mL was applied to each kg of pelleted diet. The same volume of tap water was applied to the diets that did not receive enzyme. The average xylanase activity of XYL10C was 215 U/mg of protein using a 1% solution of Azo-Xylan (Megazyme International Ltd., Wicklow, Ireland) as a substrate as per manufacturer's directions.

Eight wethers were fitted with 3.75-cm rumen cannulas (Kehl Polímeros Ltda, São Carlos, SP, Brazil), 60 d prior to the commencement of the study. Each wether was randomly assigned to one of the diets described above in each period within a replicated 4 × 4 Latin square experiment. Each period lasted for 21 d, with 14 d for adaption and the final 7 d for sample collection. Wethers were housed in metabolic crates (0.95 × 1.50 m) between 0800 h on day 15 and 0800 h on day 20. Wethers were not removed from crates during the total collection period. All other days they were housed in individual dirt floor pens (0.97 × 2.82 m) covered with wood shavings within a closed barn, conditions identical to those used in the growth performance study. Wethers were fed once daily at ad libitum at 0930 h, and water was freely available. Wethers were restricted to 95% of ad libitum intake, 2 d prior to the start of total fecal collection. Samples of feed and orts were collected and weighed daily to determine DMI. Prior to the first collection period, lambs were shorn, pre-fitted with straps to attach canvas fecal collection bags and fed in metabolic crates with transparent panels, which allowed visual contact among individuals.

Table 1. Ingredients and chemical composition of the experimental diets (± SD; *n* = 4 per diet)

	Control		AFEX[1]	
Item	Enz−[2]	Enz+[3]	Enz−	Enz+
Ingredient, % DM				
Alfalfa pellets[4]	50.0		20.0	
AFEX straw pellets[5]	—		30.0	
Barley grain	35.3		35.3	
Corn Dried distillers' grain with solubles (DDGS)[6]	5.0		5.0	
Canola meal	5.0		5.0	
Dried molasses	1.3		1.3	
Canola oil	1.0		1.0	
Sheep mineral[6]	1.0		1.0	
Urea	0.5		0.5	
Ammonium chloride	0.4		0.4	
Dicalcium phosphate	0.3		0.3	
Calcium carbonate	0.250		0.250	
Vitamin ADE	0.020		0.020	
Bovatec 20 (20% lasalocid)	0.018		0.018	
Chemical composition				
DM, %	95.9 ± 0.71	95.9 ± 0.74	96.3 ± 0.62	96.4 ± 0.55
OM, % of DM	91.5 ± 0.33	91.5 ± 0.63	93.2 ± 0.36	93.4 ± 0.40
CP, % of DM	19.6 ± 0.94	19.7 ± 0.97	16.5 ± 0.16	17.0 ± 0.56
NDF, % of DM	30.6 ± 3.85	30.5 ± 2.90	38.0 ± 1.45	36.9 ± 2.06
ADF, % of DM	21.8 ± 3.24	22.3 ± 3.61	27.9 ± 1.64	28.5 ± 1.19
NFC, % of DM	37.7 ± 2.87	37.7 ± 2.49	35.8 ± 1.15	36.6 ± 1.70
Ether extract, % of DM	3.7 ± 0.28	3.7 ± 0.47	2.9 ± 0.23	3.0 ± 0.16
Ca, % of DM	1.15 ± 0.069	1.14 ± 0.132	0.64 ± 0.087	0.66 ± 0.061
P, % of DM	0.41 ± 0.042	0.43 ± 0.040	0.37 ± 0.029	0.37 ± 0.044
NE_m, Mcal/kg	1.61 ± 0.062	1.60 ± 0.066	1.52± 0.025	1.55 ± 0.029
NE_g, Mcal/kg	1.00 ± 0.057	1.00 ± 0.055	0.93 ± 0.021	0.95 ± 0.028

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz− = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Chemical composition of alfalfa hay pellets: 89.0% OM, 17.8% CP, 54.8% NDF, 44.5% ADF, and 2.0% ether extract.
[5]Chemical composition of AFEX wheat straw pellets: 94.2% OM, 9.7% CP, 69.9% NDF, 54.4% ADF and 0.9% ether extract.
[6]Sheep mineral constituents (%): salt 92.6, potassium magnesium sulfate 4.979, zinc sulfate 0.921, magnesium sulfate 0.835, organic iodine 0.014, 1% selenium premix 0.143, cobalt carbonate 0.004, canola oil 0.398. Vitamin ADE constituents: vitamin A 10,000,000 IU, vitamin D 1,000,000 IU, vitamin E 10,000 IU/kg.

Sampling procedure

Feces and urine were collected daily throughout the first 5 d of each sample collection period (days 15 to 19). Sulfuric acid (4 M, 100 mL) was placed in urine collection containers each morning to prevent NH_3 volatilization. Fecal collection bags were emptied once daily at 0800 h. Feces that fell into the crate were incorporated in calculations of total fecal production, but not included in the subsample. Subsamples of feces (20%) and urine (10%) were taken daily and composited by wether and by period. Urine subsamples were diluted with distilled water at a ratio of 1:5 and stored at −20 °C until analyzed. Samples of the pooled feces (500 g) and diets were dried at 55 °C for 72 h to determine DM content.

Rumen pH loggers (small LRCpH, Dascor Inc., Escondido, CA) were inserted into the rumen to record pH over 5 d between 0930 h on day 15 and 0930 h on day 20, whereas wethers were housed in metabolic crates. The electrodes were calibrated at 39 °C in pH 4 and 7 buffers before insertion and set to record pH every min. Following removal from the rumen, data were downloaded and electrodes were recalibrated to account for drift in pH estimates. The pH data were standardized for each wether and summarized by day as average pH, maximum, and minimum values. Duration below and area under the curve (**AUC**) was also estimated for pH thresholds of 6.0 and 5.6. Area under the curve was the sum of the absolute value of pH below the threshold multiplied by the duration below and reported in pH × min (Ribeiro et al., 2016). Area under the curve was corrected for intake by dividing AUC by DMI.

Rumen fluid samples (250 mL) were collected on days 20 and 21, at 6 and 24 h after feeding (just prior to the morning feeding). Samples were obtained from the ventral, cranial, and caudal sacs, and mixed with fibrous material from the rumen mat. Rumen contents were strained through 2 layers of PECAP nylon (mesh opening 355 µm; Sefar Canada Inc., Ville St. Laurent, QC, Canada) to separate liquid and solid samples. Subsamples (5 mL) of rumen liquid were mixed with 1 mL of 25% (wt/vol) metaphosphoric acid and 1 mL of 1% (vol/vol) H_2SO_4 for analysis of VFA and NH_3-N, respectively. All samples were stored at −20 °C until analyzed. For enumeration of protozoa, rumen fluid (5 mL) was mixed with 5-mL methyl green formalin salt solution (Ogimoto and Imai, 1981) and stored in the dark until counted.

Rumen bacteria and archaeal diversity

Samples were removed from the rumen 24 h after feeding through the cannula and rumen contents were separated into

liquid and solid phases as described above. The solid contents were transferred to a labeled falcon tube, flash frozen in liquid N, and stored at –80 °C until processed. Samples were then freeze-dried and ground with a coffee grinder. The DNA was extracted from approximately 0.1 g of the freeze-dried, ground material using a Zymobiomics DNA extraction kit (Zymo Research, Irvine, CA). Concentration and purity of the extracted metagenomic DNA was tested by nanodrop. A PCR was conducted to amplify the full length 16S rRNA gene using the primers 27F (5′-AGAG TTTGATCMTGGCTCAG-3′) and 1398R (5′-TACGGYTACCTTGT TACGACTT-3′) to ensure that there were no PCR inhibitors in the sample.

Sequencing was performed at McGill University and Genome Quebec Innovation Center, Montreal, QC, Canada, using the Illumina MiSeq Reagent Kit v2 (500 cycle) following the manufacturer's guidelines. The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGG GTWTCTAAT-3′) targeting the V4 region of the 16S rRNA gene were used to examine bacterial and archaeal diversity. A 33 cycle PCR using 1 µL of a 1 in 10 dilution of genomic DNA and the Fast Start High Fidelity PCR System (Roche, Montreal, PQ) was conducted with the following conditions: 94 °C for 2 min, followed by 33 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final elongation step at 72 °C for 7 min. Fluidigm Corporation (San Francisco, CA) barcodes were incorporated in a second PCR using the FastStart High Fidelity PCR System under the following conditions: 95 °C for 10 min, followed by 15 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 3 min. Amplification of PCR products was confirmed in a 2% agarose gel. All samples were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and were pooled in equal proportions. Pooled samples were then purified using calibrated Ampure XP beads (Beckman Coulter, Mississauga, ON). The pooled samples (library) were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal Kit (Kapa Biosystems, Wilmington, MA). Average fragment size was determined using a LabChip GX (PerkinElmer, Waltham, MA) instrument.

Raw fastq files were imported into Qiime2 (www.qiime2.org) for sequence analysis. Primer and adapter sequences were removed from sequence files with the plugin "cutadapt" (Martin, 2011). Following removal of primer and adapter sequences, the program DADA2 (Callahan et al., 2016) was used for quality control, filtering of any phiX reads present, and removal of chimeric sequences. DADA2 models correct errors in Illumina sequence data and generates a feature table containing count data (abundance) of sequences at the strain level of resolution (>99.9% id OTUs) (Callahan et al., 2016). Following DADA2, the MAFFT program was used to perform a multiple sequence alignment, highly variable regions were masked, and a phylogenetic tree was generated with FastTree (Price et al., 2010). Taxonomy was assigned to sequences using a Naïve-Bayes classifier trained with the Silva 128 reference database and the feature-classifier plugin (Bokulich et al., 2018). Samples were subsampled to the lowest number of sequences in all samples (17,485) to ensure that α- and β-diversity analysis used the same number of sequences per sample. The diversity plugin "core-diversity-metrics" was used to assess microbial diversity within (α-diversity) and between samples (β-diversity). α-Diversity measures for richness (Shannon's diversity index), phylogenetic diversity (Faith's phylogenetic diversity), number of observed OTU, evenness (Pielou's evenness), and taxonomic abundance were evaluated. β-Diversity analysis was carried out using weighted and unweighted UniFrac (Lozupone et al., 2010). Sequences have been deposited to the Small Reads Archive (NCBI) with accession number PRJNA534318.

Growth performance study

Animals, experimental design, and diets

Sixty Canadian Arcott × Rideau Arcott lambs were used in a growth performance experiment arranged in completely randomized block design. Lambs were weighed (BW; 24.9 ± 4.32 kg) blocked by sex and weight, and randomly assigned to 1 of the 4 diets (n = 15 per diet, 8 rams and 7 ewe lambs). Lambs were housed in individual pens (0.97 × 2.82 m) within a closed barn.

Sampling procedure

Weaned lambs were adapted to individual pens and fed a pelleted diet for 7 d prior to the start of the experiment. Diets were offered for ad libitum intake at 0930 h each day for the duration of the trial with water freely available. Feed deliveries were recorded daily, with orts collected and weighed weekly to estimate weekly DMI. Dry matter content of feed and orts were determined by oven-drying samples at 55 °C for 72 h. Individual BW was recorded weekly to determine ADG by regression, and G:F was calculated as ADG:DMI.

Blood samples were collected via the jugular vein before feeding from 32 lambs (n = 8 per diet; 4 ewe and 4 ram lambs) a day prior to commencing the experiment; after 7 and 14 d and the day prior to slaughter. Blood was collected in a 6-mL vacuum tube, containing sodium heparin (BD Vacutainer REF 367878, Franklin Lakes, NJ). Plasma was obtained by centrifugation (2,000 × g for 20 min at 4 °C) and stored in a 7-mL screw-cap tube at –20 °C until analyzed for acetamide, a byproduct of AFEX treatment.

Lambs were slaughtered at a target live weight of ≥50 kg, in 2 lots (days 71 and 114) at a commercial abattoir (SunGold Specialty Meats Ltd., Innisfail, AB, Canada). Lambs that reached target weight on day 70 were included in the first lot, and those that did not were shipped in the second lot. Within 5 min of exsanguination, diaphragm (~10 g each) was collected from each lamb. Diaphragm samples were placed on ice, transported to the lab, and stored at –20 °C until analyzed for acetamide.

Hot carcass weight was recorded and grade rule (body wall thickness, mm) was determined from the total tissue depth of the carcass between the 12th and 13th rib, 11 cm from the carcass midline after the carcass had cooled for at least 30 min. Yield grades (YG) were assigned on this basis with YG1 at 5 to 11 mm, YG2 at 12 to 18 mm, YG3 at 19 to 23 mm, YG4 at 24+ mm, and C at 0 to 4 mm. Dressing percentage was calculated as carcass weight divided by the lamb live weights taken 1 d prior to shipping and multiplied by 100.

Sample analysis

Feed and fecal samples were oven dried at 55 °C for 72 h and subsequently ground through a 1-mm screen (Standard model 4; Wiley mill; Arthur H. Thomas, Philadelphia, PA). Samples were analyzed for analytical DM (AOAC, 2005; method 930.15), OM (method 942.05), NDF, and ADF. Ash content was determined by combustion of samples in a muffle furnace at 550 °C for 5 h (OM = 100 – ash). Samples were analyzed sequentially for NDF (Mertens, 2002) and ADF (AOAC, 2005; method 973.18), with modifications for use of a fiber analyzer (F57 Fiber Filter Bags, 200 Fiber Analyzer, ANKOM Technology; Vogel et al., 1999), with heat-stable α amylase (Termamyl 120, Sigma–Aldrich, St.

Louis, MO), sodium sulfite, and residual ash included in the NDF procedure.

Subsamples of dried feed and feces were ground in a ball mill (Mixer Mill MM 2000, Retsch, Haan, Germany) for determination of starch and N. For urinary N, 150 µL of diluted acidified urine was oven dried for 24 h. Nitrogen in feed, feces and urine was quantified by flash combustion with gas chromatography (**GC**) and thermal conductivity detection (Carlo Erba Instruments, Milan, Italy; AOAC, 2005; method 990.03) with CP calculated as N × 6.25. Starch was determined by hydrolyzing α-glucose polymers using a mixture of amyloglucosidase (Megazyme International Ltd., Wicklow, Ireland) and 1,4-α-D-glucan glucanohydrolase (Brennfag Canada Inc., Toronto, ON, Canada) as described by Herrera-Saldana et al. (1990). Samples were read on a Thermo Scientific Appliskan 1.437 (SkanIt Software 2.3 RE) microplate reader at a wavelength of 490 nm.

Protozoa were enumerated under a light microscope using a counting chamber (Hausser Scientific, Horsham, PA) as described by Dehority (1993). Concentration of NH_3-N in rumen samples were analyzed by the phenol-hypochlorite method as described by Broderick and Kang (1980). Rumen VFA were determined by GC (5890A Series Plus II, Hewlett Packard Co., Palo Alto, CA) with crotonic acid as an internal standard. The chromatograph was equipped with a 30-m Zebron free fatty acid phase fused silica capillary, 0.32-mm I.D. and 1.0-µm film thickness (Phenomenex, Torrance, CA), and flame-ionization detection. Oven temperature was set at 150 °C for 1 min, and then it was ramped up 5 °C/min to 195 °C, and held at this final temperature for 5 min. The carrier gas was helium (28.5 cm/s) and used as the carrier gas with the injector port at 225 °C (50:1 split) and the detector at 250 °C.

Acetamide analysis

Acetamide was quantified in AFEX wheat straw pellets following the method of Chundawat et al. (2010). In diaphragm samples, acetamide was quantified using the method of Vismeh et al. (2018). In brief, diaphragm samples were finely chopped and extracted with methanol. Propionamide was used as an internal standard based on a concentration of 0.50 µg/g meat. The meat extract was derivatized with 9-xanthydrol at 40 °C for 2.5 h. Potassium hydroxide was used to neutralize the solution and xanthydrol-derivatized acetamide was isolated in the ethyl acetate phase. After centrifugation, the collected ethyl acetate fraction was evaporated to dryness, the precipitate was resuspended in ethyl acetate, centrifuged and the supernatant transferred to vials for GC/mass spectrometry (**MS**) analysis. The GC/MS analyses were carried out at the Michigan Biotechnology Institute using an Agilent 7890N GC/MS system equipped with Agilent 7683 autosampler and a 5973C single quadrupole mass spectrometer (Agilent Technologies). A VF-5ms column (Agilent CP9013, 0.25 mm I.D., 30 m length, 0.25 µm film thickness) was used for analytical separation with helium as the carrier gas.

The method of Vismeh et al. (2018) for measuring acetamide in meat samples was slightly modified to measure acetamide in blood plasma. A 100-µL aliquot of plasma was transferred to a 2-mL Eppendorf tube, and then 15 µL of the internal standard (5 µg/mL propionamide in methanol) was added to the tube (0.5 mg/L). The reaction volume was brought up to 150 µL with water. To precipitate proteins and to reduce the pH to ~2,300 µL of 0.5 M HCl in MeOH was added to each tube. Tubes were vortexed, and then placed in a –80 °C freezer for 1 h to precipitate proteins. Tubes were centrifuged at 2,627 × *g* for 10 min. The resulting supernatant was collected and derivatized. A 250-µL aliquot of plasma extract was transferred to another Eppendorf tube, 200 µL of xanthydrol (5%) solution was added, and the mixture was incubated at 40 °C for 2 h in the dark. Plasma acetamide levels were analyzed by GC/MS as described above for the diaphragm.

Statistical analysis

Data were analyzed using the mixed model procedure of SAS (SAS Inst. Inc., Cary, NC). The univariate procedure was used to test for normality. For the metabolism study, wether was considered the experimental unit (n = 8) for all variables. The model included the fixed effects of enzyme, diet, and enzyme × diet, and the random effects of square, period within square, and wether within square. Protozoal counts were $\log_{10}$ transformed prior to statistical analysis, and duration below and AUC for pH 6.0 and 5.6 were square-root transformed. The model for the lamb performance study included enzyme, diet, and enzyme × diet as fixed effects, and sex and lamb in each treatment as random effects. False discovery rate (FDR)-corrected *P*-values were calculated using Tukey's test. The GLIMMIX procedure (SAS Inst. Inc.) was used to analyze carcass yield grade and days on feed. Differences among treatments were separated using the PDIFF option, and declared significant at $P \leq 0.05$ with trends considered at $0.05 < P \leq 0.10$.

Results

Nutrient digestibility and metabolism study

Total rumen protozoa counts were greater ($P \leq 0.04$) in wethers fed AFEX compared with the control diet 6 and 24 h after feeding, with enzyme reducing ($P \leq 0.05$) total protozoa counts in lambs fed both diets (Table 2). Before the morning feeding (24 h after feeding), ruminal NH_3-N (mM) was lower ($P = 0.004$) in wethers fed AFEX when compared with the control diet, but after 6 h, NH_3-N concentrations were greater ($P = 0.02$) for AFEX than control.

Enzyme did not affect ($P \geq 0.16$) ruminal NH_3-N at either sampling. For total VFA (mM), there was a diet × enzyme interaction 24 h after feeding ($P < 0.001$) with greater concentrations observed for the control diet with enzyme and lower concentrations observed for the AFEX diet with enzyme. There was no enzyme or diet × enzyme effect ($P \geq 0.18$) for total VFA 6 h after feeding, but lower ($P < 0.001$) concentrations were observed for AFEX when compared with the control diet. Molar proportions of ruminal acetate were greater ($P = 0.006$) 24 h after feeding, and those of propionate, valerate, isobutyrate, isovalerate, and caproate lower ($P \leq 0.03$) in lambs fed AFEX when compared with those fed the control diet. Compared with the control diet, AFEX also promoted greater ($P = 0.02$) ruminal molar proportions of acetate and lower proportions of valerate and caproate ($P \leq 0.001$) 6 h after feeding. A diet × enzyme interaction ($P \leq 0.02$) was observed for butyrate, isobutyrate, and isovalerate 6 h after feeding, with greater molar proportions observed for the control diet with enzyme, with no differences among other treatments. Enzyme decreased acetate proportions 24 h after feeding feeding ($P = 0.03$), whereas propionate proportions decreased ($P = 0.03$) 6 h after feeding and the acetate:propionate ratio tended ($P = 0.06$) to increase.

For rumen pH, a diet × enzyme interaction ($P < 0.01$) was observed for mean and max pH and for duration and AUC below pH 6 (Table 3). Enzyme increased mean and max ruminal pH for AFEX, but not for the control diet, and decreased duration and AUC below pH 6. Corrected for DMI, AFEX increased ($P = 0.01$) minimum ruminal pH, and reduced ($P \leq 0.03$) the duration,

Table 2. Effect of AFEX wheat straw and a recombinant fibrolytic enzyme on ruminal fermentation parameters, protozoa counts, and rumen pH of wethers (n = 8 per treatment)

	Control		AFEX[1]			P-value		
Item	Enz–[2]	Enz+[3]	Enz–	Enz+	SEM	Diet	Enz[4]	Diet × Enz
24 h after feeding								
Protozoa, × 10^5 per mL	6.6	5.2	18.1	8.1	0.37	0.01	0.03	0.53
pH	6.78ab	6.64b	6.70ab	6.85a	0.104	0.31	0.87	0.03
NH_3-N, mM	14.2	15.7	11.4	12.6	1.25	0.004	0.16	0.83
Total VFA, mM	66.0bc	88.2a	71.3b	54.1c	7.00	0.002	0.59	<0.001
VFA proportions, mol/100 mol								
Acetate	59.9	58.0	63.7	60.6	2.37	0.006	0.03	0.62
Propionate	22.3	21.5	20.7	20.6	1.24	0.03	0.44	0.51
Butyrate	11.0	11.5	10.5	11.2	1.10	0.350	0.21	0.88
Valerate	1.89	1.96	1.16	1.32	0.151	<0.001	0.15	0.54
Isobutyrate	1.85	1.67	1.26	1.54	0.179	0.010	0.69	0.10
Isovalerate	2.25	2.00	1.46	1.85	0.257	0.016	0.70	0.10
Caproate	0.47	0.41	0.35	0.36	0.144	0.02	0.56	0.35
Acetate:propionate	2.74	2.75	3.18	3.11	0.263	0.001	0.80	0.73
6 h after feeding								
Protozoa, × 10^5 per mL	3.1	0.9	8.7	3.6	0.21	0.04	0.05	0.70
pH	5.30	5.44	5.44	5.40	0.100	0.43	0.46	0.13
NH_3-N, mM	12.8	11.5	16.3	15.1	2.14	0.02	0.38	0.95
Total VFA, mM	169.3	154.7	136.5	135.9	11.33	<0.001	0.18	0.19
VFA proportions, mol/100 mol								
Acetate	54.7	58.0	59.4	59.0	2.73	0.02	0.22	0.11
Propionate	26.1	23.1	26.3	25.0	1.60	0.30	0.03	0.42
Butyrate	12.6b	15.6a	11.1b	10.4b	0.96	<0.001	0.15	0.02
Valerate	1.79	1.69	1.30	1.32	0.160	<0.001	0.56	0.43
Isobutyrate	0.38b	0.57a	0.31b	0.30b	0.047	<0.001	0.01	0.007
Isovalerate	0.27b	0.46a	0.23b	0.17b	0.051	<0.001	0.07	0.002
Caproate	0.48	0.36	0.25	0.25	0.111	<0.001	0.14	0.14
Acetate:propionate	2.06	2.49	2.36	2.44	0.256	0.34	0.06	0.20

[a–c]Within a row, means without a common superscript differ ($P < 0.05$).
[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz– = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).

Table 3. Effect of AFEX wheat straw and a recombinant fibrolytic enzyme on rumen pH of wethers (n = 8 per treatment)

	Control		AFEX[1]			P-value		
Item	Enz−[2]	Enz+[3]	Enz−	Enz+	SEM	Diet	Enz[4]	Diet × Enz
Ruminal pH								
Mean	5.88b	5.86b	5.84b	6.04a	0.058	0.02	0.001	<0.001
Minimum	5.39	5.36	5.41	5.47	0.049	0.01	0.51	0.07
Maximum	6.60b	6.62b	6.55b	6.78a	0.118	0.28	0.006	0.03
SD of mean pH	0.32	0.33	0.31	0.33	0.040	0.99	0.31	0.67
Duration of pH, h/d								
<6.0	17.5a	17.1a	18.1a	13.1b	1.36	0.005	<0.001	<0.001
<5.6	7.09	6.05	6.32	3.62	1.483	0.03	0.01	0.27
AUC[5], pH × h/d								
<6.0	6.2a	6.0a	5.9a	3.5b	0.84	0.001	0.001	0.007
<5.6	1.00	1.06	0.80	0.37	0.180	0.02	0.25	0.15
AUC/kg DMI, pH × h								
<6.0	4.2	4.3	3.5	2.5	0.55	<0.001	0.15	0.07
<5.6	0.83	0.84	0.60	0.42	0.131	0.03	0.49	0.45

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz− = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).
[5]AUC = area under the curve.
[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

AUC, and AUC below pH 5.6 compared with the control diet. Compared with the control diet, AFEX also reduced ($P < 0.001$) the AUC below pH 6.0 when corrected for DMI. Enzyme reduced ($P = 0.01$) the duration that ruminal pH stayed below 5.6.

Neither diet nor enzyme affected ($P \geq 0.23$) the copy number of rumen bacteria, number of OTUs, or the Shannon and Faith indices (Table 4). However, rumen bacteria evenness was reduced ($P = 0.05$) with AFEX compared with the control diet, and increased ($P < 0.01$) by enzyme.

Phylogenetic analysis identified 21 phyla within the rumen microbiota, with nince (Bacteroidetes, Firmicutes, Spirochaetes, Fibrobacteres, Actinobacteria, Proteobacteria, Kiritimatiellaeota, Synergistetes, and Planctomycetes) having a relative community sequence abundance >0.5% (Table 5). The 3 most abundant phyla were Bacteroidetes, Firmicutes, and Spirochaetes. The relative abundance of Firmicutes, Actinobacteria, and Kiritimatiellaeota in the rumen decreased ($P \leq 0.03$), and Spirochaetes increased ($P < 0.01$) in lambs fed AFEX compared with the control diet. Enzyme increased ($P < 0.001$) the relative abundance of Kiritimatiellaeota, but did not affect ($P \geq 0.14$) other phyla.

A total of 31 genera were identified with relative abundancies >0.5% (Table 6) with *Prevotella* 1, *Treponema* 2, and uncultured Prevotellaceae being the most abundant. Compared with the control diet, AFEX increased ($P < 0.01$) the relative abundancies of Lachnospiraceae NK4A136 and *Treponema* 2, and decreased ($P \leq 0.05$) that of *Succiniclasticum*, *Syntrophococcus*, *Mogibacterium*, and *Lachnoclostridium* 1. Enzyme addition to diets increased ($P \leq 0.02$) the relative abundancies of Prevotellaceae UCG-004, Christensenellaceae R-7 group, *Saccharofermentans*, and uncultured Kiritimatiellaeota, and decreased ($P \leq 0.02$) Prevotellaceae NK3B31 group and Lachnospiraceae NK3A20 group.

Digestibility of DM did not differ ($P > 0.10$) among treatments, but digestibility of CP was reduced ($P < 0.001$), and digestibility of NDF and ADF ($P \leq 0.05$) increased with AFEX compared with the control diet (Table 7). Enzyme did not affect nutrient digestibility ($P > 0.10$). There was no diet × enzyme effect ($P \geq 0.20$) on N utilization (Table 8). Compared with the control diet, AFEX tended ($P = 0.09$) to increase fecal N excretion and lower ($P = 0.01$) N retention. Enzyme tended ($P = 0.09$) to lower fecal N excretion, but did not affect ($P \geq 0.36$) other N utilization variables.

Growth performance study

Replacing alfalfa with AFEX wheat straw increased ($P = 0.003$) the DMI of lambs throughout the study and improved ($P = 0.03$) ADG in the first 42 d, but not over the full feeding period ($P = 0.51$; Table 9). Consequently, G:F of lambs was greater ($P = 0.05$) during the first 14 d, but over the entire feeding period was reduced ($P = 0.04$) with AFEX compared with the control diet (0.188 vs. 0.199). The addition of enzyme to the diet improved DMI of AFEX diets up to 70 d ($P = 0.01$), but did not affect DMI of the control diet. Enzyme improved ADG for both diets in the first 28 d ($P = 0.02$), but cumulative intervals afterwards and the overall feeding period were not affected ($P \geq 10$) by enzyme. As a result, G:F was improved with enzyme for the first 28 d ($P = 0.04$), but not overall ($P = 0.45$).

There were no differences ($P > 0.10$) among treatments in final BW, hot carcass weight, or dressing percentage (Table 9). However, days on feed were reduced ($P = 0.04$) for the AFEX when compared with the control diet. Lambs fed AFEX also tended ($P = 0.09$) to have a greater grade rule when compared with control diet. As a result, a greater proportion ($P = 0.10$) of lamb carcasses associated with this diet classified as YG3. Enzyme did not affect ($P > 0.10$) any of the carcass traits.

Table 4. Effect of AFEX wheat straw diet and a recombinant fibrolytic enzyme on α-diversity indices of bacterial communities in the rumen of wethers ($n = 8$ per treatment)

	Control		AFEX[1]			P-value		
Item	Enz−[2]	Enz+[3]	Enz−	Enz+	SEM	Diet	Enz[4]	Diet × Enz
Observed OTUs	286	239	249	285	23.3	0.77	0.76	0.23
Shannon	7.47	7.18	7.22	7.43	0.172	0.98	0.76	0.38
Evenness	0.912	0.925	0.909	0.916	0.0066	0.05	<0.01	0.21
Faith	33.5	31.2	31.6	33.4	1.88	0.90	0.83	0.62

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz− = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).

Table 5. Effect of AFEX wheat straw diet and a recombinant fibrolytic enzyme on ruminal abundance of archaea and bacteria, and phylum-level taxonomic composition of the most abundant bacterial communities (%) in the ruminal contents of wethers (n = 8 per treatment)

	Control		AFEX[1]			P-value		
Item	Enz−[2]	Enz+[3]	Enz−	Enz+	SEM	Diet	Enz[4]	Diet × Enz
Archaea	2.4	2.4	2.7	2.2	0.265	0.81	0.35	0.46
Bacteria	97.6	97.6	97.3	97.8	0.265	0.81	0.35	0.46
Bacteroidetes	46.0	44.9	50.7	47.8	3.53	0.17	0.46	0.72
Firmicutes	36.8	36.7	31.1	34.8	2.25	0.02	0.28	0.23
Spirochaetes	7.0	6.3	9.1	9.0	1.26	<0.01	0.61	0.66
Fibrobacteres	2.9	2.0	2.0	2.4	0.47	0.56	0.51	0.18
Actinobacteria	2.4	2.0	1.7	1.1	0.44	0.02	0.14	0.70
Proteobacteria	0.9	1.3	1.0	1.2	0.26	0.92	0.28	0.67
Kiritimatiellaeota	0.7	1.2	0.3	1.1	0.32	0.03	<0.001	0.12
Synergistetes	0.7	0.8	0.7	0.5	0.21	0.30	0.61	0.15
Planctomycetes	0.4	0.5	0.2	0.6	0.03	0.60	0.21	0.45
Others (<0.5%)	0.9	1.0	0.8	1.1	0.27	0.99	0.25	0.47

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz− = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).

Levels of acetamide in the blood exhibited diet × day and enzyme × day interactions ($P < 0.001$), so as a result, means were compared for each day separately (Table 10). As expected, no difference ($P > 0.05$) among treatments were observed on day 0 or just before lambs started to receive their diets. However, after 7 d, blood plasma acetamide concentrations increased in lambs fed AFEX compared with control. However, after 14 d, the levels of acetamide in the blood of lambs receiving AFEX started to decrease. The day before slaughter, concentrations of acetamide in the blood of lambs fed AFEX were much lower than during the first week, but still greater ($P < 0.05$) than in lambs fed the control diet. Acetamide concentrations in the diaphragm were greater ($P < 0.001$) in lambs fed AFEX compared with control, and reflected the higher blood acetamide concentrations prior to slaughter.

Discussion

Nutrient digestibility and metabolism study

Ammoniation of straw enhances the in vitro efficacy of exogenous fibrolytic enzymes increasing both the rate and extent of ruminal fiber degradation (Wang et al., 2004; Eun et al., 2006). We evaluated the in vivo effects of a recombinant fibrolytic enzyme specifically selected for increasing ruminal straw digestion (Ribeiro et al., 2018). This study examined whether the enzyme was equally effective against AFEX wheat straw diet and a control alfalfa hay-based pelleted diet for sheep. Contrary to our hypothesis, total tract fiber digestibility was not improved by enzyme treatment of AFEX or control diets. The rumen is considered to be the most efficient microbial system at degrading lignocellulosic biomass (Flint et al., 2008). Further improving ruminal fiber digestion is not a trivial task as evidenced by the inconsistencies in responses to fibrolytic enzymes in numerous studies with cattle and sheep (Adesogan et al., 2014; Meale et al., 2014). The enzyme used in this study (XYL10C) improved fiber digestion when tested in combination with a crude mixture of rumen enzymes in a high-throughput in vitro microassay and in in vitro batch culture experiments, but failed to improve fiber digestion in an artificial rumen (RUSITEC; Ribeiro et al., 2018). Lack of an improvement in fiber digestion in the RUSITEC is consistent with the results of the present study.

We recently showed that AFEX substantially increased the in situ NDF fractional rate of digestion (kd; 0.025 vs. 0.032 h^{-1}), total potentially degradable fraction (A+B; 59.2% vs. 81.1%), and effective rumen degradability of wheat straw (Beauchemin et al., 2019). The increase in rumen fiber degradability with AFEX can be explained by a decrease in lignin-hemicellulose ester linkages (Chundawat et al., 2011). In the present study, replacing alfalfa hay pellets with AFEX wheat straw pellets in the diet of sheep increased NDF and ADF content, reduced CP content, with no effect on DM digestibility. Interestingly, the digestibility of NDF and ADF was increased in AFEX when compared with alfalfa diets, despite the higher fiber content of the AFEX diet.

Branched-chain VFA (i.e., valerate, isovalerate and isobutyrate) in the rumen primarily originate from ruminal oxidative-deamination and decarboxylation of valine, leucine, and isoleucine (Tedeschi et al., 2000). Hence, the lower molar proportions of the branched-chain VFA in the rumen of wethers fed AFEX compared with the control diet aligns with the lower CP content of the diet and the lower CP digestibility observed. The increase in acetate concentrations in the rumen of wethers

Table 6. Effect of AFEX wheat straw diet and a recombinant fibrolytic enzyme on genus-level taxonomic composition of the most abundant bacterial communities (%) in the ruminal contents of wethers (n = 8 per treatment)

		Control		AFEX[1]			P-value		
Phylum	Genus[2]	Enz−[3]	Enz+[4]	Enz−	Enz+	SEM	Diet	Enz[5]	Diet × Enz
Bacteroidetes									
	Prevotella 1	18.44	17.42	19.62	18.00	2.544	0.65	0.51	0.88
	Prevotellaceae	6.91	4.97	6.52	8.84	3.024	0.48	0.94	0.39
	Paraprevotella	2.36	1.41	1.47	3.79	1.225	0.46	0.50	0.11
	Rikenellaceae RC9 gut group	2.23	1.42	2.10	2.03	0.439	0.51	0.25	0.32
	Prevotellaceae_UCG-001	2.15	1.89	1.81	1.86	0.284	0.50	0.69	0.54
	Prevotellaceae_UCG-003	1.99	1.89	2.30	2.01	0.335	0.43	0.48	0.72
	Muribaculaceae uncultured	1.48	1.13	1.27	0.95	0.372	0.49	0.25	0.96
	Prevotellaceae_NK3B31_group	1.46	0.93	1.50	0.66	0.346	0.68	0.02	0.57
	Prevotellaceae_UCG-004	0.75	0.97	0.51	1.26	0.229	0.90	0.02	0.20
	Prevotella 7	0.87	0.56	0.86	0.40	0.086	0.79	0.31	0.82
Firmicutes									
	Lachnospiraceae NK3A20 group	4.08	3.58	3.88	3.15	0.011	0.20	0.02	0.59
	Ruminococcus 1	2.25	1.30	1.26	2.29	0.343	0.99	0.91	0.14
	Acetitomaculum	2.02	1.44	2.10	1.43	0.035	0.94	0.16	0.92
	Lachnospiraceae	1.74	1.67	1.21	1.87	0.355	0.57	0.32	0.23
	(Ruminococcus) gauvreauii group	1.70	1.57	1.15	1.30	0.305	0.18	0.99	0.64
	Christensenellaceae R-7 group	1.50	4.12	0.77	4.53	0.895	0.80	<0.001	0.41
	Succiniclasticum	1.32	1.37	0.80	0.65	0.197	<0.001	0.73	0.53
	Ruminococcus 2	1.15	0.53	0.55	0.80	0.236	0.48	0.43	0.08
	Syntrophococcus	1.10	0.72	0.44	0.49	0.013	0.02	0.44	0.27
	Ruminococcaceae NK4A214 group	0.92	1.21	1.18	1.41	0.247	0.34	0.28	0.91
	Mogibacterium	0.82	0.75	0.53	0.50	0.141	0.03	0.67	0.86
	Veillonellaceae	0.73	1.00	0.47	0.72	0.027	0.29	0.29	0.95
	Lachnoclostridium 1	0.70	0.78	0.38	0.64	0.005	0.02	0.09	0.28
	Moryella	0.69	0.56	0.45	0.43	0.014	0.15	0.59	0.70
	Saccharofermentans	0.67	1.54	0.37	1.79	0.043	0.73	<0.01	0.35
	[Eubacterium] coprostanoligenes group	0.62	0.42	0.43	0.50	0.113	0.57	0.51	0.17
	Lachnospiraceae NK4A136 group	0.29	0.22	0.47	0.88	0.028	<0.01	0.34	0.11
	Other genera of Firmicutes (<0.5%)	10.58	9.58	7.90	8.70	0.901	0.02	0.71	0.17
Spirochaetes									
	Treponema 2	6.95	6.00	9.06	8.84	1.246	<0.01	0.47	0.64
Fibrobacteres									
	Fibrobacter	2.92	2.01	2.04	2.36	0.469	0.56	0.51	0.18
Actinobacteria									
	Olsenella	1.17	0.87	0.79	0.51	0.326	0.16	0.27	0.97
	Other genera of Actinobacteria (<0.5%)	1.19	1.10	0.62	0.57	0.253	0.01	0.73	0.91

Table 6. Continued

Phylum	Genus[2]	Control		AFEX[1]		SEM	P-value		
		Enz–[3]	Enz+[4]	Enz–	Enz+		Diet	Enz[5]	Diet × Enz
Kiritimatiellaeota									
Kiritimatiellaeota uncultured		0.54	0.88	0.10	0.73	0.209	0.05	<0.01	0.30
Kiritimatiellaeota others (<0.5%)		0.09	0.24	0.09	0.25	0.020	0.96	0.12	0.99

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Only the genera with relative abundance > 0.5% in at least one group and the were significantly affected by treatments were presented.
[3]Enz– = diet without enzyme.
[4]Enz+ = diet with enzyme.
[5]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).

Table 7. Effect of AFEX wheat straw and a recombinant fibrolytic enzyme on apparent total tract digestibility of wethers (n = 8 per treatment)

Item	Control		AFEX[1]		SEM	P-value		
	Enz–[2]	Enz+[3]	Enz–	Enz+		Diet	Enz[4]	Diet × Enz
DMI, kg/d	1.42	1.41	1.59	1.33	0.122	0.64	0.21	0.25
Digestibility, %								
DM	69.6	69.3	67.8	67.6	1.93	0.20	0.85	0.97
OM	72.2	71.6	69.5	69.5	1.87	0.09	0.81	0.81
CP	74.3	73.9	69.4	69.8	1.16	<0.001	0.99	0.67
NDF	45.6	42.1	51.1	48.9	3.84	0.05	0.32	0.81
ADF	46.0	42.1	50.0	50.6	3.72	0.03	0.52	0.39

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz– = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).

Table 8. Effect of AFEX wheat straw diet and a recombinant fibrolytic enzyme on nitrogen (N) utilization by wethers (n = 8 per treatment)

	Control		AFEX[1]			P-value		
Item	Enz−[2]	Enz+[3]	Enz−	Enz+	SEM	Diet	Enz[4]	Diet × Enz
N intake, g/d	44.8	44.3	41.9	38.1	3.69	0.16	0.50	0.61
Fecal N, g/d	11.7	10.3	12.9	11.7	1.01	0.10	0.09	0.92
Urine N, g/d	17.5	19.9	18.3	17.6	1.61	0.56	0.48	0.20
RN[5], g/d	14.4	14.4	9.8	10.3	2.57	0.01	0.87	0.84
RN/N intake, %	31.0	32.1	21.1	25.4	4.35	0.02	0.36	0.58
RN/digested N, %	41.5	41.6	30.6	36.5	5.55	0.05	0.41	0.41
RN, g/kg $BW^{0.75}$	0.69	0.74	0.48	0.52	0.122	0.02	0.53	0.98

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz− = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).
[5]RN = retained N.

fed AFEX is likely a result of increased fiber degradation in the rumen, an observation that aligns with the greater NDF digestibility of this diet.

The greater NH_3-N concentration in the rumen of wethers fed the control diet compared with AFEX just prior to the morning feeding (24 h after feeding) is consistent with the higher total CP content (17.8% vs. 9.7%) and higher concentration of true protein in alfalfa compared with AFEX wheat straw. Recently, Beauchemin et al. (2019) showed that CP of wheat straw increased from 2.5% to 10.3% with AFEX treatment. Although, AFEX can more than double the CP content of straw (9.9% vs. 4.3%; Chundawat et al., 2013), this increase is due to ammonia becoming bound to the straw and the formation of other nitrogenous compounds such as acetamide. Thus, the increase in CP is due to an increase NPN, which can account for 56% to 76% of total N in AFEX straw (Chundawat et al., 2013; Beauchemin et al., 2019). The NPN content of alfalfa hay has been shown to range from 13.1% to 28.8% of total N (Sniffen et al., 1992; Lines and Weiss, 1996). The greater ruminal NH_3-N concentration observed for wethers fed AFEX diets 6 h after feeding is consistent with the greater NPN of AFEX, as NPN is rapidly solubilized and converted to NH_3-N in the rumen.

Rumen protozoa decline as the pH falls below 5.6, and are generally eliminated when ruminal pH reaches 5.3 to 5.4 (Dehority, 2003). In the present study, the greater concentrations of protozoa with AFEX compared with the control diet is consistent with the higher minimum ruminal pH (5.44 vs. 5.38) and lower duration that the ruminal pH was below 5.6 (0.59 vs. 1.03 h/d). These results are consistent with the higher fiber content observed for AFEX when compared with control diets (37.4% vs. 30.5% NDF), which may have stimulated greater salivation and consequently promoted increased ruminal buffering (Pitt et al., 1996; Kolver and de Veth, 2002). The residual ammonia in AFEX wheat straw may also promote an alkalizing effect on ruminal pH, contributing to the higher minimum pH observed for the AFEX when compared with the control diet.

Chemical composition and physical structure of the diet are considered the factors that most affect the rumen microbiome (Henderson et al., 2015). In this study, replacing 30% of alfalfa hay with AFEX wheat straw decreased ruminal bacterial diversity (evenness) and decreased Firmicutes, Actinobacteria, and Kiritimatiellaeota, while increasing Spirochaetes. Firmicutes abundance has been shown to increase with increasing fiber content in the diet, and decrease during the transition from a forage to a high-concentrate diet (Petri et al., 2013; Nathani et al., 2015; Kittelmann et al., 2015). However, the AFEX diet promoted lower Firmicute abundance despite an increase in the fiber content of the diet. The decrease in Firmicutes in wethers fed AFEX was compensated for by an increase in the abundance of Spirochaetes, particularly *Treponema*. Spirochaetes are also assumed to play a key role in the degradation of complex fiber (Paster and Canale-Parola, 1982). Initial studies by Blackburn and Hungate (1963) demonstrated that co-culturing *Treponema* with *Fibrobacter succinogenes* enhanced the ability of this bacterium to degrade barley straw as *Treponema* decarboxylated succinate. However, recent studies support a more direct action of *Treponema* on pectin and cellulose degradation (Rosewarne et al., 2012; Liu et al., 2014; Newbrook et al., 2017). Analysis of the bacteria attached to rice straw and alfalfa over time within the rumen showed that the relative abundance of *Treponema* peaked between 16 and 48 h of incubation (i.e., second colonizers), confirming that it plays a specific role in plant fiber degradation (Liu et al., 2016). However, the relative abundance of *Treponema*

Table 9. Effect of AFEX wheat straw diet and a recombinant fibrolytic enzyme on intake, production performance, and carcass characteristics of finishing lambs (n = 15 per treatment)

	Control		AFEX[1]			P-value		
Item	Enz−[2]	Enz+[3]	Enz−	Enz+	SEM	Diet	Enz[4]	Diet × Enz
DMI, kg/d								
0 to 14 d	1.12b	1.04b	1.15b	1.27a	0.087	0.001	0.54	0.007
0 to 28 d	1.21c	1.15c	1.30b	1.43a	0.110	<0.001	0.28	0.003
0 to 42 d	1.29c	1.21c	1.39b	1.51a	0.117	<0.001	0.53	0.002
0 to 56 d	1.35c	1.27c	1.45b	1.56a	0.121	<0.001	0.66	0.002
0 to 70 d	1.42b	1.34b	1.50b	1.59a	0.135	<0.001	0.80	0.01
0 to end	1.52	1.47	1.58	1.63	0.088	0.003	0.92	0.13
ADG, g/d								
0 to 14 d	294b	273b	329b	394a	33.7	<0.001	0.27	0.04
0 to 28 d	302	318	325	368	39.7	0.004	0.02	0.27
0 to 42 d	306	301	326	327	38.7	0.03	0.82	0.76
0 to 56 d	308	295	324	316	42.5	0.07	0.31	0.79
0 to 70 d	308	298	318	311	44.3	0.21	0.38	0.86
0 to end	301	295	309	300	51.1	0.51	0.45	0.90
G:F								
0 to 14 d	0.259	0.276	0.288	0.316	0.0178	0.05	0.21	0.77
0 to 28 d	0.249	0.278	0.249	0.257	0.0118	0.23	0.04	0.26
0 to 42 d	0.236ab	0.252a	0.234ab	0.217b	0.0111	0.01	0.96	0.01
0 to 56 d	0.226a	0.236a	0.218a	0.202b	0.0121	<0.001	0.52	0.02
0 to 70 d	0.216a	0.222a	0.212a	0.195b	0.0120	0.003	0.27	0.03
0 to end	0.197	0.200	0.193	0.183	0.0223	0.04	0.45	0.21
Initial BW, kg	25.4	24.0	24.3	25.3	2.69	0.91	0.86	0.20
Final BW, kg	53.0	51.7	51.4	52.0	2.00	0.58	0.74	0.43
DOF[5], d	97	97	91	91	—	0.04	1.00	1.00
HCW[6], kg	23.8	23.2	23.6	23.9	2.28	0.66	0.80	0.51
Dressing percentage	45.6	45.8	46.0	46.0	0.57	0.58	0.86	0.87
Grade rule, mm	15.2	15.3	16.9	16.4	1.55	0.09	0.77	0.70
Grade[7]								
YG1, %	6.7	20.0	6.7	6.7	—	0.55	0.55	0.55
YG2, %	66.7	60.0	40.0	46.7	—	0.30	0.95	0.72
YG3, %	26.7	20.0	53.3	46.7	—	0.10	0.65	0.87

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz− = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).
[5]DOF = number of days on feed.
[6]HCW = hot carcass weight.
[7]Yield grade based on grade rule measurements at least 30 min after cooling. YG1 = 5 to 11 mm, YG2 = 12 to 18 mm, YG3 = 19 to 23 mm. YG1 is optimal and declining to YG3.
[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

Table 10. Effect of AFEX wheat straw diet and a recombinant fibrolytic enzyme on acetamide concentrations (mg/kg) in blood and diaphragm of lambs ($n = 8$ per treatment)

	Control		AFEX[1]			P-value		
Item	Enz−[2]	Enz+[3]	Enz−	Enz+	SEM	Diet	Enz[4]	Diet × Enz
Acetamide in blood								
Day 0	0.57[a]	0.83[a]	0.96[a]	0.62[a]	1.516	<0.001	0.39	<0.001
Day 7	1.32[b]	16.09[a]	40.22[a]	30.78[a]				
Day 14	1.09[c]	1.58[c]	30.83[a]	6.16[b]				
1 d before slaughter	1.01[b]	1.32[b]	9.50[a]	9.87[a]				
Acetamide in diaphragm	1.83	1.47	8.27	11.88	1.154	<0.001	0.73	0.08

[1]AFEX = ammonia fiber expansion treatment (30% AFEX straw pellets on a dietary DM basis replacing alfalfa hay pellets).
[2]Enz− = diet without enzyme.
[3]Enz+ = diet with enzyme.
[4]Enz = recombinant fibrolytic enzyme treatment effect (XYL10C, a β-1,4-xylanase, EC 3.2.1.8, GH10 from *Aspergillus niger*).
[a–c]Within a row, means without a common superscript differ ($P < 0.05$).

associated with alfalfa declined after 16 to 48 h of ruminal incubation, whereas it remained high for rice straw (Liu et al., 2016). This difference seems to be associated with the faster rate of fiber digestion of alfalfa compared with rice straw, as by 48 h of incubation most of the alfalfa would be digested or passed out of the rumen. In our study, rumen samples for microbial analysis were collected ~24 h after feeding when most of the alfalfa fiber may already have been digested when compared with AFEX wheat straw. This would support the higher relative abundance of *Treponema* observed for wethers fed AFEX when compared with the control diet.

Kiritimatiellaeota is a newly described phylum that was previously classified as Verrucomicrobia, but little is known about its members (Spring et al., 2016). Spring et al. (2016) showed that *Kiritimatiella glycovorans* L21-Fru-AB^T fermented xylose to ethanol and acetate, but was not able to utilize starch, cellobiose, galactose, maltose, sucrose, or fructose. The relative abundance of Kiritimatiellaeota in our study could be related to differences in the structural composition of the fiber in alfalfa as a dicot compared with AFEX wheat straw as a monocot. The primary cell wall of dicots is richer in xyloglucan and pectic polysaccharides compared with monocots (Åman, 1993), and the degradation of these substrates in the rumen may promote the release of greater amounts of xylose to support the growth of Kiritimatiellaeota.

Pretreatment of feed with enzymes before feeding or incubation with ruminal fluid has been shown to improve the ability of these additives to enhance ruminal fermentation in several studies (Lewis et al., 1999; Wang et al., 2001; Ribeiro et al., 2018). Several in vitro (Wang et al., 2001; Yang et al., 2002; Giraldo et al., 2008) and in vivo (Tirado-Estrada et al., 2011; Bhasker et al., 2013; Wang et al., 2018) studies have shown that fibrolytic enzymes can alter the molar proportions of VFA, but shifts in the pattern of VFA are inconsistent across studies and seem to be influenced by the nature of the feed and enzyme used. Although total tract fiber digestibility was not affected by enzyme in our study, some changes in the rumen microbiota and fermentation parameters were observed. The decrease in molar proportions of acetate 24 h after feeding is difficult to explain as this enzyme increased the molar proportion of acetate in batch culture studies (Ribeiro et al., 2018). This difference between in vitro and in vivo studies may be related to enzyme induced shifts in rumen microbiota in vivo as shown by the increased ruminal bacterial diversity (evenness) and decreased protozoa counts. The decrease in protozoa population is consistent with a reduction in acetate, as acetate and butyrate are the main VFA produced during fermentation of starch and cellulose by protozoa (Morgavi et al., 1994). Interestingly, the relative abundance of the Christensenellaceae R-7 group increased (1.13% vs. 4.33%) with enzyme treatment. Christensenellaceae has been shown to have an important role in biofilm formation and rumen degradation of starch and fiber (Mao et al., 2015; De Mulder et al., 2017), producing acetic and butyric acid from the fermentation of glucose (Morotomi et al., 2012). Enzyme also increased the relative abundance of *Saccharofermentans* (0.52% vs. 1.67%), uncultured Prevotellaceae_UCG-004 (0.63% vs. 1.11%), and the phylum Kiritimatiellaeota (0.46% vs. 1.15%), while decreasing Prevotellaceae NK3B31 group (1.48% vs. 0.80%) and Lachnospiraceae NK3A20 group (3.98% vs. 3.37%). The increase in the Kiritimatiellaeota is consistent with the ability of XYL10C, a β-1,4-xylanase to increase the release of xylose residues from hemicellulose. These findings support previous observations by Wang et al. (2001), where they suggested that pretreatment of feed with fibrolytic enzymes could change the species profile

of colonizing rumen bacteria. The pretreatment of feeds with enzymes is suggested to cause the release of reducing sugars and other hydrolysis products, promoting chemotactic response in ruminal microbes, and stimulating their attachment to feed particles (Cheng and McAllister, 1997; Beauchemin et al., 2003; Giraldo et al., 2007). Not only is the profile of bacteria attached to feed modified, but previous studies have also reported an increase in bacterial attachment as a result of pretreatment of forages with enzymes (Wang et al., 2001; Ribeiro et al., 2015, 2018). Differences in the amount and profile of bacteria attached to feedstuffs during ruminal digestion as a result of pretreatment with enzymes may also partially explain the variation in responses reported in some studies (Adesogan et al., 2014; Meale et al., 2014).

Growth performance study

The increase in ADG up to day 42 suggests that there was an initial benefit of feeding the AFEX diet compared with the control alfalfa diet, but this response was not maintained throughout the feeding period. This increase in ADG was likely a result of the higher DMI in lambs fed AFEX when compared with those fed alfalfa. This may be a consequence of the greater total tract fiber digestibility, which may have promoted lower gut fill with the AFEX diet compared with the control diet. Gain:feed for the full feeding period was reduced by 5.1% for lambs fed AFEX when compared with the control diet, a response possibly reflected by the tendency for lower OM digestibility, ruminal propionate concentration, and N retention in wethers fed these diets in the metabolism study. Although the N retention (g/kg $BW^{0.75}$) was 30.6% lower in wethers fed AFEX when compared with those fed the control diet in the metabolism study, there was no difference in the weight gain of AFEX and control lambs in the growth performance study. The decreased N retention was partially compensated for by the greater DMI (6.6%) of lambs fed AFEX. Willms et al. (1991) observed that lambs fed alkaline hydrogen peroxide-treated wheat straw diets had maximum microbial protein synthesis when fed 17% of the supplemental CP from NPN in the form of urea, and that when more than 33% CP was provided by NPN it decreased total amino acid flow to the duodenum and lamb performance. In the present study, NPN of AFEX accounted for ~34% of the total dietary CP. In addition, most fiber-degrading microorganisms in the rumen require branched-chain VFA for growth (Van Gylswyk, 1970; Bryant, 1973). The rumen microbes use branched-chain VFA as a source of carbon skeletons to synthesize branched-chain amino acids (Allison et al., 1962a,b). The lower quality of the protein and greater NPN as a proportion of total dietary CP with AFEX compared with alfalfa may have resulted in lower branched-chain VFA concentrations in the rumen of lambs. This most likely reduced the efficiency of microbial protein synthesis in the rumen and prevented greater N retention and ADG in lambs fed AFEX. However, the reduction in days on feed for lambs fed AFEX compared with the control diet (91 vs. 97 d) may offset the cost of the lower G:F with AFEX.

Grade rule is a measurement of body wall thickness across the lean, bone, and subcutaneous fat between the 12th and 13th rib, 11 cm from the carcass midline. The accumulation of subcutaneous fat in this area, which increases the grade rule, is an indicator of expected trimmed cut yield from the carcass. The tendency for greater grade rule and consequent greater proportions of lamb carcasses classified as YG3 for AFEX compared with control may be explained by greater ruminal production of acetate, the main substrate used for the synthesis of subcutaneous adipose tissue in ruminants (Ingle et al., 1972).

Fibrolytic enzyme improved the ADG and G:F of lambs for the first 28 d of the study, but this beneficial effect was not maintained throughout the feeding period. This suggests that the enzyme may have promoted growth in lambs early in the feeding period, but not after they were fully adapted to the diets. The improvements in fiber digestion observed by Ribeiro et al. (2018) with this same enzyme in short-term rumen batch cultures, but not in RUSITEC would also support this hypothesis. The reduction in the duration that the ruminal pH was below 5.6 with enzyme treatment of diets may also support the improved performance observed for lambs in the first month of the feeding period before they were fully adapted to the diets.

Hydrolysis of ester cross-links between hemicellulose-xylan chains and lignin polymers by ammonia treatment may produce ionized amide groups (Tarkow and Feist, 1969). During AFEX treatment, acetamide (CH_3CONH_2) is produced (Weimer et al., 2003) as the simplest amide derived from acetic acid. Acetamide is currently present in the food chain in products such as milk, beef and thermally processed foods such as roasted coffee beans (Vismeh et al., 2018). High levels of acetamide in the diet (2.36%) have been shown to increase the incidence of liver carcinoma in rats (Jackson and Dessau, 1961; Fleischman et al., 1980), resulting in it being classified as Group 2B human carcinogen by the International Agency for Research on Cancer (Vismeh et al., 2018). A recent study suggested that some specific rumen bacteria can metabolize acetamide to acetate and ammonia preventing it from accumulating in the meat or milk of ruminants (Mor et al., 2019). Our results agree with Mor et al. (2019) and suggest that the ability of rumen microbes to metabolize acetamide increased within 2 wk of feeding AFEX wheat straw. However, concentrations of acetamide in the diaphragm of lambs after slaughter were still greater for AFEX (10.1 mg/kg) compared with the control treatment (1.7 mg/kg), although well below that which was found to cause cancer in rats (Fleischman et al., 1980). Interestingly, concentrations of acetamide in blood plasma of lambs 1 d before slaughter were very similar to the concentrations in the diaphragm just after slaughter, suggesting that blood plasma levels may be a good predictor of acetamide concentrations in muscle.

The LD50 of oral administration of acetamide to rats was determined to be 7,000 mg/kg of BW (Kegley et al., 2016). No information about LD50 as a result of oral administration of acetamide has been generated for other animals. The concentration of acetamide in the AFEX-treated pellets (5,600 mg/kg) suggests that it will not reach lethal levels even if the LD50 for rats is used as a reference for ruminants. In addition, the concentration of acetamide in the complete diet (1,680 mg/kg) in the present study was 14-fold lower than the concentration of acetamide in the diet (23,600 mg/kg) that promoted liver carcinoma in rats (Fleischman et al., 1980). Because rumen microorganisms are able to adapt and partially degrade acetamide, it is to be expected that the carcinogenic concentration and LD50 of oral administration of acetamide in ruminants is much higher than for rats. Average concentrations of acetamide in regular pasteurized milk, beef, and roasted coffee beans are 0.4, 0.4, and 39.0 mg/kg, respectively (Vismeh et al., 2018). Even though concentrations of acetamide were increased in the diaphragm of lambs fed AFEX, the concentrations were still substantially lower (10.1 mg/kg) than that in other common foods and are unlikely to pose a risk to human health.

The digestibility and performance observed for wethers and lambs fed AFEX straw compared with alfalfa confirm that AFEX technology has the potential to increase the nutritional value of low-quality roughages. The greater digestibility of NDF and

ADF of AFEX compared with the control diet demonstrates how potent this physical/chemical treatment is as this would not have been possible if untreated wheat straw was included in the diet.

In conclusion, AFEX-treated wheat straw pellets can partially replace alfalfa pellets in the diet of growing lambs, with minimal impact on growth performance. The DMI of lambs fed AFEX slightly increased, but no differences in ADG were observed when compared with an alfalfa pellet-based diet, resulting in a slight decrease in feed efficiency. Acetamide concentration in blood plasma initially increased due to inclusion of AFEX wheat straw in the diet, but declined over the feeding period, suggesting adaptation of lambs to AFEX and rumen metabolism of acetamide. Pretreatment of feed with the enzyme XYL10C decreased rumen protozoal counts and altered the bacterial populations in the rumen digesta, but feed efficiency was only improved in the first 28 d of the feeding period.

Acknowledgments

The financial support for this study from Elanco Animal Health and the AIP program of Agriculture and Agri-Food Canada is gratefully acknowledged. The authors thank the Lethbridge Research and Development Centre (AAFC) staff for their technical and animal care assistance. We also recognize the Michigan Biotechnology Institute (MBI) for suppling AFEX-treated wheat straw. The authors thank Drs. Farzaneh Teymouri, Bryan Bals, and Ms. Dianne Haddad from MBI for preparing AFEX-treated wheat straw and for conducting the acetamide analysis.

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