Effect of exogenous fibrolytic enzymes and ammonia fiber expansion on the fermentation of wheat straw in an artificial rumen system (RUSITEC)¹

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ABSTRACT: This study investigated the effect of treatment of wheat straw using ammonia fiber expansion (AFEX) and exogenous fibrolytic enzymes (Viscozyme) on fiber digestibility, rumen fermentation, microbial protein synthesis, and microbial populations in an artificial rumen system [Rumen Simulation Technique (RUSITEC)]. Four treatments were assigned to 16 vessels (4 per treatment) in 2 RUSITEC apparatuses in a randomized block design. Treatments were arranged as a 2 × 2 factorial using untreated or AFEX-treated wheat straw with or without exogenous fibrolytic enzymes [0 or 500 µg of protein/g straw dry matter (DM)]. Fibrolytic enzymes were applied to straw, prior to sealing in nylon bags. The concentrate mixture was provided in a separate bag within each fermentation vessel. The RUSITECs were adapted for 8 d and disappearance of DM, neutral detergent fiber (NDF), acid detergent fiber (ADF), and crude protein (CP) was measured after 48 h of incubation. Ammonia fiber expansion increased ($P < 0.01$) the disappearance of wheat straw DM (69.6 vs. 38.3%), NDF (65.6 vs. 36.8%), ADF (61.4 vs. 36.0%), and CP (68.3 vs. 24.0%). Total dietary DM, organic matter (OM), and NDF disappearance was also increased ($P \leq 0.05$) by enzymes. Total microbial protein production was

greater ($P < 0.01$) for AFEX-treated (72.9 mg/d) than untreated straw (63.1 mg/d). Total gas and methane (CH₄) production ($P < 0.01$) were also greater for AFEX-treated wheat straw than untreated straw, with a tendency for total gas to increase ($P = 0.06$) with enzymes. Ammonia fiber expansion increased ($P < 0.01$) total volatile fatty acid (VFA) production and the molar proportion of propionate, while it decreased ($P < 0.01$) acetate and the acetate-to-propionate ratio. The AFEX-treated straw had lower relative quantities of fungi, methanogens, and *Fibrobacter succinogenes* ($P < 0.01$) and fewer protozoa ($P < 0.01$) compared to untreated straw. The pH of fermenters fed AFEX-treated straw was lower ($P < 0.01$) than those fed untreated straw. Both AFEX ($P < 0.01$) and enzymes ($P = 0.02$) decreased xylanase activity. There was an enzyme × straw interaction ($P = 0.02$) for endoglucanase activity. Enzymes increased endoglucanase activity of AFEX-treated wheat straw, but had no effect on untreated straw. The addition of enzymes lowered the relative abundance of *Ruminococcus flavefaciens*, but increased *F. succinogenes*. These results indicate that AFEX increased the ruminal disappearance of wheat straw and improved fermentation and microbial protein synthesis in the RUSITEC.

Key words: AFEX, fibrolytic enzymes, fiber, rumen fermentation, RUSITEC

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INTRODUCTION

Production of cereal crop residues, such as cereal straws and corn stover, has increased dramatically as a result of more cereal grain production. The Food and Agriculture Organization (FAO, 2004) estimated that about 2 billion tonnes of these residues are produced annually. Despite the widespread availability of these lignocellulosic residues, their poor digestibility limits their use in ruminant diets (Hatfield et al., 1999; Azzaz et al., 2012). Improving fiber digestibility of these crop residues could provide ruminants in many regions of the world with a higher value feed source.

Cellulose, hemicellulose, and lignin are the major components of the plant cell wall (Rubin, 2008). Ester linkages between hemicellulose and lignin along with acetyl and ferulic acid side groups limit plant cell wall digestion (Sun et al., 2002; Wyman et al., 2005; Nishimura et al., 2018). Thus, physical (Wyman et al., 2005; Wang et al., 2012), chemical (Wang et al., 2004; Peterson et al., 2015), and enzymatic (Wang et al., 2004; Eun et al., 2006) approaches have been assessed for their ability to improve plant cell wall digestion.

Most commercial exogenous enzymes are extracts of bacterial (e.g., *Bacillus*) or fungal (e.g., *Aspergillus*, *Trichoderma*) origin (Muirhead, 1996). Numerous studies have examined the ability of exogenous enzymes to improve the utilization of fibrous feeds (Mendoza et al., 2014). Exogenous enzymes have been reported to have positive effects (Adesogan et al., 2014; Valdes et al., 2015; Mohamed et al., 2017) and no effects (Adesogan et al., 2007; Baloyi, 2008; Ribeiro et al., 2015) on the digestibility of fiber.

Among chemical treatments, ammonia fiber expansion (AFEX), originally developed for cellulosic ethanol production, shows considerable potential to improve the digestibility of crop residues for ruminants (Bals et al., 2010). This chemical process cleaves the ester bonds between lignin and hemicellulose, as well as ether linkages between lignin and other polymeric carbohydrates (Buranov and Mazza, 2008; Chundawat et al., 2010). Evaluating corn stover and late-harvest switchgrass, AFEX treatment improved 48 h in vitro neutral detergent fiber (NDF) digestibility by 52% and 128% as compared to untreated samples (Bals et al., 2010).

A number of studies have been conducted to evaluate the effects of combining exogenous enzymes with chemical treatment on the digestibility of cereal straws. Synergistic responses between ammonia pretreatment and exogenous enzymes have been reported to improve the digestibility of rice straw (Wang et al., 2004; Eun et al., 2006; Wang et al., 2012). We hypothesize that a synergistic response between AFEX and exogenous enzymes will further enhance the digestibility of crop residues. The objective of this study was to evaluate whether addition of exogenous fibrolytic enzymes to AFEX-treated wheat straw enhances fermentation, digestibility, and microbial protein synthesis in Rumen Simulation Technique (RUSITEC).

MATERIALS AND METHODS

Experimental Design and Treatments

The experiment was designed as a completely randomized block, with 4 treatments ($n = 4$ per treatment) arranged in a 2×2 factorial and randomly assigned to 16 fermentation vessels in 2 RUSITECs. Treatments included AFEX-treated and untreated wheat straw with or without exogenous fibrolytic enzymes. The 15-d experiment consisted of 8 d for adaptation and 7 d for sample and data collection. The diet consisted of 70% wheat straw and 30% concentrate (DM basis; Table 1), and was formulated to meet or exceed protein, mineral, and vitamin requirements of beef heifers weighing 450 kg and gaining 1.0 kg/d [National Academies of Sciences, Engineering, and Medicine (NASEM), 2016]. Supplementing the diet with concentrate ensured that fiber degradation was not limited by protein, mineral, or vitamin deficiencies. Substrates (7 g straw and 3 g concentrate; DM basis) were ground through a 4-mm screen using a Wiley mill (Arthur Thomas Co., Philadelphia, PA) and weighed into 2 separate nylon bags (10×20 cm and 5×10 cm; pore size of 50 μ m, Ankom Technology Corp., Macedon, NY). The concentrate mixture composition (DM basis) was 66.7% corn dried distillers grains with solubles, 26.6% canola meal, 4.2% calcium carbonate, 1% of urea, 0.8% dicalcium phosphate, 0.5% salt, 0.19% feedlot premix, and 0.01% vitamin E.

Table 1. Ingredient and chemical composition of substrates

Item	Ingredients		
	Untreated wheat straw	AFEX ¹ -treated wheat straw	Concentrate ²
Chemical composition ³ , % of DM			
DM	91.8	93.4	94.0
OM	92.1	94.9	89.4
CP	3.19	8.42	35.7
EE	1.5	1.2	7.2
NDF	80.0	66.6	37.4
ADF	47.9	48.9	16.3
NFC	7.4	18.7	9.1

¹AFEX = ammonia fiber expansion.

²Composition (DM basis): 66.7% of corn dried distillers grains with solubles, 26.6% of canola meal, 4.2% calcium carbonate, 1% of urea, 0.8% dicalcium phosphate, 0.5% salt, 0.19% feedlot premix, and 0.01% vitamin E.

³DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; NFC = non-fiber carbohydrate.

A commercial enzyme mixture (Viscozyme L) was obtained from Novozymes Inc. (Copenhagen, Denmark). The enzyme mixture was produced from a strain of *Aspergillus aculeatus* and contained a range of carbohydrases, including arabanase, cellulase, beta-glucanase, hemicellulose, xylanase, and pectinase (Gama et al., 2015). According to the manufacturer, the principal activity was ≥ 100 beta-glucanase U/g. Fibrolytic enzymes were applied only to straw at 500 μ g protein/g DM. Enzymes were sprayed over the straw using a hand sprayer and it was incubated at 39 °C for 18 h before being placed in the RUSITEC. The dosage was based on our previous study (Ribeiro et al., 2018) and enzymes were diluted in water (1 mL) so as to deliver the desired enzyme concentration directly onto straw before sealing in nylon bags. The same amount of water was applied to control straw.

AFEX Pretreatment

Ammonia fiber expansion treatment of wheat straw was undertaken at the Michigan Biotechnology Institute (Lansing, MI) using packed bed AFEX reactors as described by Campbell et al. (2013). Briefly, wheat straw was ground through a 30.5-mm screen and packed into stainless steel baskets at a density of 100 kg/m² prior to insertion into reactor tubes. Baskets were pre-steamed to displace air and raise the temperature of the straw to 80 to 85 °C. Ammonia vapor was introduced into the reactor tubes at a rate of 80–100 g/min to a level of 1 kg ammonia per kilogram of straw. The pressure

in the vessel was increased to 200 psi and after 30 min, the pressure was rapidly released. Residual ammonia was removed and recovered by steam stripping and used to treat the next batch of straw.

Inoculum Source and Experimental Apparatus

Inocula were obtained from 3 ruminally cannulated Angus cross cows [averaging body weight (BW) 769 $\pm$ 11.2 kg] fed a basal diet of 60% barley silage, 30% straw, and 10% protein, mineral, and vitamin supplement (DM basis). Cows were handled in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009), and protocols were reviewed and approved by the Lethbridge Research and Development Centre Animal Care Committee. Rumen fluid was collected 2 h after the morning feeding from 4 distinct sites within the rumen, strained through 4 layers of cheese cloth, and equally pooled by site and animal. Inoculum from each cow (4 L per cow) was mixed together, pH was recorded, and it was kept in a water bath at 39 °C until adding to fermenters.

Two RUSITEC apparatuses (Czerkawski and Breckenridge, 1977), each equipped with eight 920-mL anaerobic fermenters, were used. Each fermenter was outfitted with an input port for infusion of buffer, and an outlet port to collect effluent. To maintain anaerobic conditions, each fermenter was flushed with CO₂ during feed-bag exchange. On the first day, each fermenter was filled with 200 mL of artificial saliva, 700 mL of strained rumen inoculum, 1 bag containing 20 g of mixed wet solid rumen digesta so as to inoculate the system with feed-particle associated rumen bacteria, and 2 bags, one containing 7 g of straw and one containing 3 g of concentrate (DM basis). Fermenters were kept in a circulating water bath at 39 °C and bags within the vessels were gently moved up and down within the fermenters at 8 cycles/min. The nylon bag containing solid rumen contents was replaced after 24 h with one containing straw and the other containing concentrate. Thereafter, 2 bags were replaced daily at 0900 h, so that each bag remained in each fermenter for 48 h. Artificial saliva (McDougall, 1948) was adjusted to pH of 8.2 and contained NaHCO₃ 9.83 g/L, NaH₂PO₄ 3.69 g/L, NaCl 0.47 g/L, KCl 0.60 g/L, CaCl₂·2H₂O 0.03 g/L, MgCl₂·6H₂O 0.06 g/L, and (NH₄)₂SO₄ 0.30 g/L. The buffer was continuously infused into fermenters using a peristaltic pump set to achieve a dilution rate of 2.9%/h, replacing 70% of the fermenter volume daily. Effluent was collected daily into a 2.0 L Erlenmeyer flask and fermentation gases were

collected into reusable 2-L gas-tight collection bags (CuriryR; Covidien Ltd., Mansfield, MA).

Nutrients Disappearance and Gas Production

Dry matter, organic matter (OM), acid detergent fiber (ADF), NDF, and crude protein (CP) disappearance from 48 h straw and concentrate incubated bags was determined from day 9 to 15. Nylon bags were withdrawn from each fermenter and washed under cold tap water until the water was clear. Bags were oven dried at 55 °C for 48 h (AOAC, 1995; method 930.15) to determine DM disappearance. Residues were pooled over 5 d, ground through a 1-mm screen using a Wiley mill (standard model 4; Arthur Thomas Co.) and analyzed for OM, N, NDF, and ADF. Ash content was determined by combustion at 550 °C for 5 h, and OM content was calculated as 100 minus the proportion of ash (AOAC, 1995; method 942.05). The NDF and ADF contents were determined using the sequential method with an ANKOM200 Fiber Analyzer (Ankom Technology Corp.) using reagents as described by Van Soest et al. (1991), with heat-stable α -amylase (Termamyl 120 L, Novo Nordisk Biochem, Franklinton, NC) and sodium sulfite included in the NDF analysis. Total N was determined using a combustion analyzer (NA 2100, Carlo Erba Instruments, Milan, Italy), with CP calculated as $N \times 6.25$. Organic matter, NDF, ADF, and CP disappearance was determined as the difference between the amount of these components in the substrate before incubation and that remaining in the residue after incubation.

Daily total gas production was measured in the morning during bag exchange using a gas meter (Model DM3A, Alexander-Wright, London, UK). A subsample of gas (20 mL) was collected from each gas bag using a syringe and injected into evacuated 6.8 mL exetainers (Labco Ltd., Wycombe, Bucks, UK). Methane (CH_4) concentration in the gas was determined using a Varian 4900 gas chromatograph equipped with GS-CarbonPLOT 30 m $\times$ 0.32 mm $\times$ 3 μm column and thermal conductivity detector (Agilent Technologies Canada Inc., Mississauga, ON, Canada) at an isothermal oven temperature of 35 °C with helium as the carrier gas (27 cm/s).

Fermentation Characteristics

Fermenter fluid pH (Orion model 260A, Fisher Scientific, Toronto, ON, Canada) and effluent volume from each fermenter were recorded daily at the time of bag exchange. Effluent (5 mL) was

sampled for analysis of volatile fatty acids (VFAs) and preserved with 1 mL of 25% (wt/wt) metaphosphoric acid, and frozen at -20 °C until analyzed. An additional 5 mL of effluent was collected, and preserved with 1 mL of H_2SO_4 (1% vol/vol) for determination of ammonia nitrogen ($\text{NH}_3\text{-N}$) as described by Rhine et al. (1998). The VFAs were quantified using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA) equipped with a capillary column (30 m $\times$ 0.32 mm i.d., 1- μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA), and flame ionization detector and crotonic acid (trans-2-butenic acid) as an internal standard. The concentration of VFA was multiplied by daily effluent production (L/d) to determine VFA production (mmol/d).

Protozoa Counts

Samples for enumeration of protozoa were collected from day 11 to 15. At the time of bag exchange, 5 mL of liquid was gently squeezed from the 48-h straw and concentrate bags and preserved in 5.0 mL (1:1 v/v) of methyl green formalin-saline solution. The samples were stored in the dark at room temperature until counted by light microscopy using a Levy-Hausser counting chamber (Hausser Scientific, Horsham, PA). Each sample was counted twice and if the duplicates differed by more than 10%, counts were repeated.

Microbial Protein Synthesis

Bacteria in the fermenters were labeled using ^{15}N as described by Saleem et al. (2018). On day 9, 0.3 g/L ^{15}N -enriched $(\text{NH}_4)_2\text{SO}_4$ (Sigma Chemical Co., St. Louis, MO; minimum ^{15}N enrichment 1 g/L) was added to replace $(\text{NH}_4)_2\text{SO}_4$ in the McDougall's buffer and infused until the end of the experiment. From day 9, daily effluent accumulation in each flask was preserved with 3 mL of a sodium azide to achieve a final concentration of 0.1% wt/vol. On day 13, 14, and 15, the daily total effluent for each fermenter was measured and a subsample (35 mL) was centrifuged ($20,000 \times g$, 30 min, 4 °C) for isolation of liquid-associated bacteria. The resulting pellets were washed using phosphate buffer and centrifuged 3 times ($20,000 \times g$, 30 min, 4 °C) prior to suspension in distilled water, freezing, and lyophilization for determination of N and ^{15}N .

Feed particle-associated and feed particle-bound bacterial fractions were measured from 48-h feed residues on day 13, 14, and 15 by squeezing solids to expel excess liquid from the nylon bags.

Straw and concentrate bags were placed together in a plastic bag with 20 mL of phosphate buffer and processed for 60 s in a Stomacher 400 Laboratory Blender (Seward Medical Ltd., London, UK). The processed liquid was squeezed out, poured off and retained. Feed residues were washed twice with 10 mL of phosphate buffer in each wash. The washed buffer was retained and pooled with the initially expressed fluid to obtain the feed particle-associated bacterial fraction, and the total volume was recorded. Washed solid feed residues were considered to represent the feed particle-bound bacterial fraction. The feed particle-associated bacterial samples collected after stomaching were centrifuged ($500 \times g$, 10 min, 4 °C) to remove large feed particles and the supernatant was decanted and centrifuged ($20,000 \times g$, 30 min, 4 °C) to isolate a bacterial pellet which was washed 3 times as described above. The pellet was then resuspended in distilled water and stored at -20 °C for determination of N and ^{15}N . Washed feed residues were dried at 55 °C for 48 h, weighed for DM determination, ball ground (MM400; Retsch Inc., Newtown, PA) and analyzed for total N and ^{15}N by combustion analysis using a mass spectrometer (NA1500, Carlo Erba Instruments).

Total effluent microbial N (MN) production (mg/d) was calculated using the N concentration (%) determined for the microbial pellet, multiplied by the microbial weight in the total effluent (mg/d). Microbial weight in the total effluent was calculated by multiplying daily effluent production (mL) by the microbial density (mg/mL) in the 35 mL subsample. Microbial N production from feed particle-associated fraction was calculated by multiplying the N concentration (%) in the feed particle-associated microbial pellet by the microbial weight of the feed particle-associated fraction (mg/d). Production of feed-particle-bound MN (mg/d) was calculated using the following equation:

$$\text{MN} = (\text{APE in RN} / \text{APE in MN}) * \text{RN}$$

where APE in RN = the percent excess of ^{15}N in the residue nitrogen, and APE in feed particle-associated microbial pellet was used as the source of APE in MN. The APE values were corrected for the natural ^{15}N background concentration observed in the substrates. Total MN production (mg/day) was calculated as the sum of MN production in the effluent, feed particle-associated and feed particle-bound bacterial fractions of the straw residue, and feed particle-bound bacterial fraction of the concentrate residue.

Efficiency of microbial protein synthesis was calculated as the ratio of MN (mg) to fermented OM (g).

Enzyme Activity

Xylanase and endoglucanase activities from days 13, 14, and 15 were determined in the supernatant of feed particle-associated samples after centrifuging at $20,000 \times g$ for 30 min at 4 °C. Sample (500 μL) was combined with 0.5 mL substrate solution (2% suspension (wt/vol) of beech wood or medium-viscosity carboxy-methyl cellulose in 0.2 M phosphate buffer, pH 6.5, for measurement of xylanase and endoglucanase activity, respectively (Miller, 1959). Samples were incubated at 50 °C, with shaking, for 10 min and the reaction was terminated by adding 3 mL 3,5-dinitrosalicylic acid and placing the tubes in boiling water for 5 min. Xylose and glucose were used to prepare standard curves for xylanase and CMCase activity, respectively. Enzyme activity was defined as the amount of enzyme required for releasing 1 μmol equivalent of xylose or glucose per minute per gram of enzyme.

DNA extraction and real-time PCR. Total DNA was extracted from all ground feed particle-associated bacterial samples using a Qiagen QIAamp DNA Stool mini kit (Qiagen, Valencia, CA) according to manufacturer instructions. Approximately, 30 mg of each sample was suspended in 1.4 mL of stool lysis buffer with 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm), and homogenized for 3 min at 30/s using a Qiagen Tissue Lyser II (Oss et al., 2016). The suspension was heated at 95 °C and mixed gently in a thermomixer (Eppendorf-Thermomixer comfort, Eppendorf Ltd., Mississauga, ON, Canada) for 5 min. Total DNA was eluted in 200 μL of Buffer AE (Elution buffer; Qiagen Inc.) and quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen Canada Inc., Burlington, ON, Canada) with a NanoDrop 3300 fluorometer (Thermo Scientific, Wilmington, DE).

Real-time PCR was used to quantify total bacteria (16S rRNA), total methanogens (*mcrA*), and total fungi (18S rRNA) as well as 16S rRNA sequences specific to *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Selenomonas ruminantium*. Primers and PCR conditions for each assay are presented in [Supplementary Data](#). Real-time PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems,

Foster City, CA). Standards and samples were assayed in a 20 μ L reaction mixture containing 10 μ L SsoAdvanced Universal SYBR Green supermix (Biorad, Richmond, CA), 0.3 μ L of the forward primer, 0.3 μ L of the reverse primer, 7.4 μ L of molecular grade water, and 2 μ L of the standard or DNA sample. Reaction conditions for each assay were 5 min of 95 °C followed by 35–40 cycles of 30 s at 94 °C, 30 s at the specific annealing temperature for each assay, and 30 sec at 72 °C with a final extension time of 5 min at 72 °C. A standard curve for each bacterial species, total bacteria, fungi, and methanogens was constructed using plasmid DNA containing the target sequence as an insert that was either amplified or isolated from a rumen DNA sample and confirmed by Sanger sequencing. Relative quantification was used in which the amplification of specific targets was reported relative to the amplification of the total bacteria 16S rRNA.

Statistical Analysis

Data were analyzed as repeated measures according to a completely randomized block design using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The MIXED model included the fixed effects of straw, enzyme treatment, and straw $\times$ enzyme treatment interaction, with RUSITEC apparatus included as a random effect and the day of sampling as a repeated measure. For the repeated measures, various covariance structures were tested with the final structure chosen based on the minimum Akaike's information criteria value (Wang and Goonewardene, 2004). Data were tested for normality of variance prior to analysis. Differences among treatments were tested using the PDIF option and significance was declared at $P \leq 0.05$ and a trend at $0.05 < P < 0.10$ unless otherwise stated. The correlation matrix generated for microbial populations was generated using the corrplot function (Wei and Simko, 2017) in R Statistics 3.4.4 (R Core Team, 2013).

RESULTS

Characteristics of AFEX Wheat Straw

Ammonia fiber expansion-treated straw was noticeably darker in color, and had a greater CP (8.42% vs. 3.19%) and ADF (48.9% vs. 47.9%) concentration, and a lower NDF concentration (66.6% vs. 80.0%) than untreated wheat straw. The non-fibre carbohydrate content of straw was also dramatically increased by AFEX (Table 1).

Nutrient Disappearance and Microbial Protein Synthesis

Disappearance of DM (69.6% vs. 38.3%), OM (69.9% vs. 36.9%), NDF (65.6% vs. 36.8%), ADF (61.4% vs. 36.0%), and CP (68.3% vs. 24.0%) in AFEX-treated wheat straw was increased ($P < 0.01$) compared to untreated wheat straw (Table 2). However, disappearance of these nutrients in the concentrate portion of the diet was greater ($P < 0.05$) in fermenters receiving untreated straw as compared to AFEX-treated straw. Compared to untreated straw, the AFEX-treated straw had greater ($P < 0.01$) total dietary DM (70.2% vs. 50.8%), OM (72.0% vs. 50.3%), NDF (66.0% vs. 43.5%), and ADF (61.8% vs. 40.9%), and lower ($P = 0.02$) total dietary CP (70.6% vs. 75.6%) disappearance. Furthermore, DM, OM, and NDF disappearance in the straw portion of the diet and disappearance of these nutrients in the total diet increased ($P \leq 0.05$) with enzyme inclusion. Moreover, there was no effect of enzyme inclusion or a straw $\times$ enzyme interaction ($P > 0.05$) for ADF or CP disappearance among treatments. Ammonia fiber expansion-treated wheat straw increased ($P < 0.01$) MN production in the effluent (39.9 vs. 31.5 mg/d) and feed particle-bound bacterial fractions of the concentrate residue (2.16 vs. 1.56 mg/d) and total MN production (72.9 vs. 63.1 mg/d). No difference was found for MN production in feed particle-bound and feed particle-associated bacterial fractions with straw. The MN production in the feed particle-associated fraction was decreased with enzyme inclusion ($P = 0.03$), but no straw $\times$ enzyme interaction was detected. Additionally, there was no effect of enzyme addition ($P = 0.34$) or straw $\times$ enzyme interaction ($P = 0.75$) for total MN production.

Ruminal Fermentation Characteristics

Ammonia fiber expansion treatment of straw increased ($P < 0.01$) total gas production and CH_4 as mg/d or mg/g of incubated DM, but there was no effect on CH_4 produced as mg/g of DM digested (Table 3). Moreover, total gas production ($P = 0.06$) and production of $\text{NH}_3\text{-N}$ ($P = 0.07$) tended to increase with enzyme inclusion. No difference in $\text{NH}_3\text{-N}$ production was found among treatments. The media pH in fermenters fed AFEX-treated straw was lower ($P < 0.01$) than in those fed untreated straw (6.67 vs. 6.83). Ammonia fiber expansion increased the production of total VFA and the molar proportions of propionate ($P < 0.01$), butyrate ($P < 0.01$), and caproate ($P = 0.09$), while

Table 2. Effect of fibrolytic enzymes and AFEX treatment of wheat straw on nutrient disappearance and microbial N production in the RUSITEC

Item	Treatment ¹				SEM	<i>P</i> -value		
	Untreated straw		AFEX-treated straw			Straw	ENZ	Int
	–ENZ	+ENZ	–ENZ	+ENZ				
Nutrient disappearance ² , %								
DM								
Wheat straw	38.1	38.4	68.7	70.5	0.95	<0.01	0.02	0.14
Concentrate	78.6	80.5	71.0	72.6	3.11	<0.01	0.16	0.93
Total	50.4	51.2	69.3	71.1	1.57	<0.01	0.02	0.40
OM								
Wheat straw	36.3	37.6	68.8	71.0	1.18	<0.01	0.04	0.46
Concentrate	80.7	83.3	75.6	79.0	2.40	0.02	0.09	0.82
Total	49.5	51.0	70.7	73.3	1.46	<0.01	0.03	0.54
NDF								
Wheat straw	36.4	37.1	64.6	66.6	1.99	<0.01	0.05	0.30
Concentrate	76.1	77.4	65.9	69.7	3.04	<0.01	0.30	0.59
Total	43.1	43.9	64.8	67.2	2.10	<0.01	0.05	0.28
ADF								
Wheat straw	36.0	36.1	60.1	62.6	2.38	<0.01	0.37	0.54
Concentrate	72.5	74.6	62.2	66.7	3.38	0.01	0.35	0.74
Total	40.7	41.0	60.4	63.1	2.27	<0.01	0.12	0.20
CP								
Wheat straw	26.7	21.4	67.0	69.6	2.06	<0.01	0.48	0.06
Concentrate	85.1	87.4	71.0	72.1	5.62	<0.01	0.43	0.86
Total	75.1	76.0	69.6	71.5	4.00	0.02	0.46	0.82
Microbial N production ³ , mg/d								
LAB ⁴	30.27	32.82	39.68	40.17	2.14	<0.01	0.79	0.89
FPA ⁵	10.69	9.41	10.95	10.24	0.46	0.26	0.03	0.51
FPB ⁶ straw	20.51	19.33	19.76	21.61	1.13	0.38	0.70	0.10
FPB concentrate	1.57	1.54	2.16	2.15	0.10	<0.01	0.84	0.95
Total	62.45	63.71	71.57	74.14	2.97	<0.01	0.34	0.75
EMP ⁷	14.32	13.37	11.34	10.89	0.54	<0.01	0.17	0.61

¹–ENZ = without enzyme; +ENZ = with enzyme; Int = interaction: straw × enzyme.

²Samples from day 11 to 15.

³Samples from day 17 to 18.

⁴LAB = liquid-associated bacteria.

⁵FPA = feed particle-associated bacteria.

⁶FPB = feed particle-bound bacteria.

⁷EMP = efficiency of microbial protein, mg microbial N production/g OM fermented.

it decreased ($P < 0.01$) the molar proportions of acetate, valerate, isobutyrate, and isovalerate. Consequently, AFEX decreased ($P < 0.01$) the acetate:propionate ratio (2.23 vs. 2.74). Molar proportions of butyrate, isobutyrate, and isovalerate were increased ($P < 0.01$) by adding exogenous enzymes. Addition of fibrolytic enzymes did not influence fermenter pH.

Enzyme Activity

There was an enzyme × straw interaction ($P = 0.02$) for endoglucanase activity, with enzyme addition increasing endoglucanase activity of

AFEX-treated straw ($P = 0.03$), with no effect on untreated straw (Table 4). Ammonia fiber expansion decreased xylanase activity (3.00 vs. 3.66 μmol of xylan/min/mL; $P < 0.01$) in feed particle-associated bacteria. Furthermore, xylanase activity decreased ($P = 0.02$) with enzyme inclusion for both treated and untreated straw.

Microbial Populations

The AFEX treatment resulted in lower ($P < 0.01$) absolute quantities of bacteria, fungi, methanogens, and protozoa and in lower ($P < 0.01$) relative quantities of fungi, methanogens, and

Table 3. Effect of fibrolytic enzymes and AFEX treatment of wheat straw on gas production and fermentation in the RUSITEC

Item	Treatment ¹				SEM	<i>P</i> -value		
	Untreated straw		AFEX-treated straw			Straw	ENZ	Int
	−ENZ	+ENZ	−ENZ	+ENZ				
Gas production, L/d	1.31	1.41	1.74	1.86	0.06	<0.01	0.06	0.87
CH ₄ , % of gas ²	6.48	6.41	7.10	7.20	0.76	<0.01	0.92	0.68
CH ₄ , mL/d	85.00	95.48	125	134	14.27	<0.01	0.15	0.95
CH ₄ , mg/d	55.25	62.06	80.95	87.21	9.28	<0.01	0.15	0.95
CH ₄ , mg/g incubated DM	5.51	6.19	8.07	8.69	0.92	<0.01	0.15	0.95
CH ₄ , mg/g digested DM	11.23	12.08	11.51	12.12	1.28	0.83	0.32	0.87
pH	6.81	6.84	6.69	6.65	0.02	<0.01	0.95	0.07
NH ₃ -N, ³ mg/d	68.30	71.62	70.31	76.83	4.92	0.18	0.07	0.55
Total VFA ⁴ , mmol/d	38.12	38.25	52.72	54.59	1.92	<0.01	0.43	0.49
VFA, mol/100 mol								
Acetate (A)	65.30	64.91	61.89	61.73	1.38	<0.01	0.33	0.68
Propionate (P)	24.01	23.81	28.49	27.83	1.64	<0.01	0.14	0.41
Butyrate	6.36	6.87	6.74	7.15	0.15	<0.01	<0.01	0.58
Valerate	1.85	1.88	1.45	1.49	0.03	<0.01	0.21	0.81
Isobutyrate	0.98	1.03	0.73	0.77	0.03	<0.01	0.01	0.87
Isovalerate	1.41	1.59	0.75	0.93	0.11	<0.01	<0.01	0.92
Caproate	0.010	0.008	0.015	0.028	0.009	0.09	0.45	0.25
A:P ratio ⁵	2.72	2.75	2.19	2.26	0.20	<0.01	0.18	0.58

¹-ENZ = without enzyme; +ENZ = with enzyme; Int = interaction: straw × enzyme.

²CH₄ = methane.

³NH₃-N = ammonia nitrogen.

⁴VFA = volatile fatty acid.

⁵A:P ratio = acetate:propionate ratio.

Table 4. Effect of fibrolytic enzymes and AFEX treatment of wheat straw on enzyme activity in the RUSITEC

Item	Treatment ¹				SEM	P-value		
	Untreated straw		AFEX-treated straw			Straw	ENZ	Int
	–ENZ	+ENZ	–ENZ	+ENZ				
Enzyme activities (μmol/min/mL)								
Endoglucanase ²	0.116 ^{b3}	0.115 ^b	0.117 ^b	0.137 ^a	0.005	0.02	0.03	0.02
Xylanase ⁴	3.80	3.51	3.07	2.92	0.12	<0.01	0.02	0.46

¹-ENZ = without enzyme; +ENZ = with enzyme; Int = interaction: straw × enzyme.

²Activity was expressed as μmol of glucose/min/mL of sample incubated at 50 °C and pH 6.5 using carboxymethylcellulose as the substrate.

³Letters a, b, and c: values with different letters differ at $P > 0.05$.

⁴Activity was expressed as μmol of xylan/min/mL of sample incubated at 50 °C and pH 6.5 using xylose as the substrate.

F. succinogenes (Table 5). The AFEX treatment had no effect on the relative quantities of *R. albus* and *R. flavefaciens* and resulted in a greater ($P < 0.01$) relative quantity of *S. ruminantium*. The addition of the fibrolytic enzymes resulted in a decline ($P < 0.01$) in *R. flavefaciens*, but had no effect on the absolute or relative quantities of the other bacterial or archaeal targets. There was a strong positive correlation ($P < 0.05$) between the relative quantities of methanogens and *F. succinogenes* and a strong negative correlation ($P < 0.05$) between the relative

quantities of *S. ruminantium* and *F. succinogenes* and methanogens (Fig. 1).

DISCUSSION

Characteristics of AFEX-Treated Wheat Straw

Crude protein concentration of AFEX-treated wheat straw was about 62% greater than untreated straw due to the presence of residual non-protein N (NPN) as a result of ammoniation. The increased

Table 5. Effect of fibrolytic enzymes and AFEX treatment of wheat straw on the relative quantities of rumen microbes

Item	Treatment ¹				SEM	P-value		
	Untreated straw		AFEX-treated straw			Straw	ENZ	Int
	–ENZ	+ENZ	–ENZ	+ENZ				
Absolute quantities ²								
Total bacteria 16S rRNA copies, ×10 ⁷	8.43	8.91	6.09	6.05	1.30	<0.01	0.76	0.73
Total fungi 18S rRNA copies	2,009	1,721	<200	<200	425	<0.01	0.78	0.69
Total methanogens, <i>mcrA</i> copies, ×10 ⁵	4.57	5.54	2.17	1.95	1.39	<0.01	0.54	0.33
Total protozoa ³ , ×10 ³ cells/mL	35.17	38.70	11.86	12.7	2.57	< 0.01	0.35	0.56
Relative quantities ⁴								
Total fungi 18S rRNA, ×10 ^{–4} %	21.0	24.0	<3.25	<3.25	5.169	<0.01	0.57	0.94
Total methanogens <i>mcrA</i> , %	0.525	0.591	0.349	0.319	0.103	<0.01	0.69	0.29
<i>Fibrobacter succinogenes</i> , %	0.643 ^{ab5}	1.49 ^a	0.165 ^{bc}	0.052 ^c	0.357	<0.01	0.20	0.03
<i>Ruminococcus albus</i> , %	0.271	0.193	0.281	0.265	0.053	0.15	0.06	0.21
<i>Ruminococcus flavefaciens</i> , %	0.131	0.091	0.110	0.072	0.014	0.12	<0.01	0.93
<i>Selenomonas ruminantium</i> , %	0.039	0.034	0.076	0.092	0.023	<0.01	0.70	0.47

¹-ENZ = without enzyme; +ENZ = with enzyme; Int = interaction: straw $\times$ enzyme.

²Absolute quantities are the average quantities of the target before normalization based on 16S rRNA total bacteria target.

³Total protozoa counts are based on counts done by light microscopy and not quantitative PCR.

⁴Relative quantities of the target are the average populations calculated as a percentage of the total bacterial 16S rRNA.

⁵Letters a, b, and c, values with different letters differ at $P > 0.05$.

CP concentration following AFEX treatment agrees with the findings of [Bals et al. \(2010\)](#) and [Blümmel et al. \(2018\)](#) who found that the CP concentration of AFEX-treated wheat straw and corn stover increased from 6.2% to 16.1%. Moreover, AFEX altered fiber structure as it reduced the NDF concentration of wheat straw by about 17% and increased the non-fiber carbohydrate (NFC) fraction by over 100%. Others have found that AFEX treatment of wheat straw reduced NDF concentration by 4.7% ([Blümmel et al., 2018](#)) to as much as 11.0% ([Bals et al., 2010](#)). [Eun et al. \(2006\)](#) reported that CP concentration of ammoniated rice straw increased by about 4% and NDF and hemicellulose concentration was reduced by 5.6% and 3.2%, respectively.

Nutrient Disappearance and Bacterial Protein Synthesis

The greater nutrient disappearance of AFEX-treated straw was consistent with the increase in total gas and VFA production, and highlights that AFEX has the potential to increase availability of nutrients in lignocellulosic feeds. In the RUSITEC, [Griffith et al. \(2016\)](#) also reported an increase in DM, OM, NDF, ADF, and CP disappearance of AFEX-treated barley straw as compared to untreated straw. [Bals et al. \(2010\)](#) reported that AFEX improved in vitro NDF disappearance of wheat straw by 19.7% after 48 h of incubation. In the present study, AFEX altered the structure

of lignocellulose by lowering NDF concentration, increasing the NFC fraction and improving NDF digestibility. The increased CP disappearance for AFEX-treated straw could be due to the easier access of ruminal microbes and enzymes to plants cell wall components as a result of improved NDF digestibility ([Graham and Åman, 1984](#); [Bals et al., 2010](#)). This in turn leads to an increase in the extent and rate of glucose and xylose release during enzymatic hydrolysis compared to untreated crop residues ([Teymouri et al., 2005](#); [Wyman et al., 2005](#)). Ammonia fiber expansion-treated straw contained more N due to ammoniation, resulting in the higher initial N concentrations as compared to untreated straw (8.42% vs. 3.29%, respectively). This NPN could still be used as a source of N to support microbial protein synthesis.

It is interesting to note that although enzymes were not applied to the concentrate portion of the diet, its disappearance was higher in untreated straw than in AFEX-treated straw. The recalcitrant nature of untreated wheat straw appeared to promote a greater ruminal digestion of the concentrate portion of the diet. This finding may reflect an increase in the bacterial population colonizing the concentrate portion of the diet as a result of the low fermentability of untreated straw in the RUSITEC fermenters ([Ribeiro et al., 2018](#)). Similar to the rumen, microbial pools or subpopulations in the RUSITEC are in a dynamic equilibrium where microbes are constantly flowing between pools

Figure 1. Correlation matrix to visualize the relationships between the relative quantities of microbial targets. The areas of the circles represent the absolute value of corresponding correlation coefficients and the colors and shading represent the direction, negative (red), and positive (blue) of the correlation. The “X” represents correlations that are not statistically significant ($P > 0.05$). Relative quantity of total fungi (18S/16S), total methanogens (mcrA/16S), *Fibrobacter succinogenes* (Fsucc/16S), *Ruminococcus albus* (Ralbus/16S), *Ruminococcus flavefaciens* (Rflav/16S), and *Selenomonas ruminantium* (Srumin/16S) were correlated to one another.

(Czerkawski and Cheng, 1988). This flow of microbes between the different pools supports our theory that changes in the microbes attached to the straw can change the microbial population in the liquid fraction and consequently in the population associated with the concentrate fraction of the diet (Ribeiro et al., 2018). The greater total MN production with AFEX-treated straw indicates that it promoted the growth and attachment of ruminal microorganisms to feed as it increased the liquid-associated bacterial fraction MN production, the concentrate feed particle-bound bacterial fraction, MN production, and endoglucanase activity as compared to untreated straw. Ammoniation cleaves the ester and ether linkages within the lignin-carbohydrate complex, reducing the physical enmeshment of cellulose and solubilizing phenolic compounds. This facilitates microbial colonization and their access to the cell wall matrix and accelerates the rate of fiber digestion (Kerley et al., 1985; Fahey et al., 1993; Nsereko et al., 2000; Wang et al., 2012). Compared to untreated straw, Wang et al. (2012) reported that ammoniated straw increased in vitro batch culture DM digestibility, gas production, total VFA production, and microbial protein synthesis.

Exogenous fibrolytic enzymes were added to wheat straw with the hypothesis that they would improve fiber degradation by stimulating the attachment and activity of rumen microorganisms (Nsereko et al., 2002) as a result of alterations in

fiber structure (Giraldo et al., 2004). A number of studies reported that exogenous fibrolytic enzymes have been shown to improve the digestibility and utilization of high fiber diets (Eun and Beauchemin, 2007; Wang et al., 2012). A commercial enzyme complex (Viscozyme L) was selected based on our previous study (Badhan et al., 2018), where Viscozyme with rumen mixed enzymes synergistically increased the saccharification efficiency of barley straw. In this same study, the enzyme activities within Viscozyme that were responsible for this synergistic response were identified (Badhan et al., 2018). The present study showed that addition of enzymes to AFEX-treated straw enhanced the disappearance of DM, OM, and NDF, but had no effect on untreated straw. Lignin and complex side chains that are associated with hemicellulose can limit the physical access of microbial enzymes to their substrates (Chesson, 1988; O’Sullivan, 1997; Hall et al., 2010). AFEX disrupts the plant cell wall matrix as it reduces the crystallinity of cellulose, alters lignin, partially hydrolyzes hemicellulose, and disrupts the ester linkages between lignin and hemicellulose and other polymeric carbohydrates (Buranov and Mazza, 2008; Yang and Wyman, 2008; Chundawat et al., 2010). This increases the availability of fermentable sugars (Mosier et al., 2005; Taherzadeh and Karimi, 2008) and the access of enzymes to cellulose and hemicellulose, improving ruminal digestion (Dale et al., 1996; Teymouri et al., 2005). Addition of exogenous

fibrolytic enzymes to ammoniated rice straw (Eun et al., 2006) or barley straw (Wang et al., 2012) synergistically improved the microbial digestion of straw. The synergistic response observed between enzymes and AFEX with wheat straw could be due the removal of these structural barriers, which in turn improves the effectiveness of exogenous enzymes to increase the availability of soluble carbohydrates to microorganisms (Wang and McAllister, 2002). Addition of enzyme to ammoniated barley straw strongly increased the rate and extent of DM digestibility in situ and in vitro, suggesting that phenolic barriers that impede the microbial digestion of the plant cell wall were removed by combining enzymes with ammoniated straw (Wang et al., 2004).

The effect of exogenous enzymes on digestibility may have been influenced by several factors, including straw type, time of incubation, and application methods (Wang et al., 2012). Several studies suggest that addition of exogenous enzymes before incubation could increase degradation of fiber through hydrolysis of the feed in vitro (Nsereko et al., 2000; Wallace et al., 2001; Giraldo et al., 2007). In the present study, the exogenous enzymes were applied to the straw 18 h prior to incubation and this may have improved the enzyme adhesion to the plant cell wall and enhanced the fermentation of AFEX straw. The pretreatment of substrates with enzymes has been proposed to increase the attachment of rumen bacteria to feed particles (Wang et al., 2001; Giraldo et al., 2007; Ribeiro et al., 2015). Moreover, Mendoza et al. (2014) hypothesized that production responses to exogenous fibrolytic enzymes in ruminant diets was dependent on feed quality, particularly those feeds composed of high levels of rumen digestible NDF. In our study, AFEX decreased the NDF concentration of straw, which contributed to an increase in rumen digestible NDF (Bals et al., 2010). This could account for the positive effect of enzymes on DM, OM, and NDF disappearance of AFEX-treated straw.

Ruminal Fermentation Characteristics

Gas production reflects the extent of ruminal microbial fermentation and it is highly correlated to DM digestibility (Menke et al., 1979). In the current study, the increasing total gas production corresponded to increases in DM and fiber disappearance with AFEX-treated straw. These results align with those of Blümmel et al. (2018) where AFEX increased in vitro gas production and the digestibility of rice straw and sorghum stover.

Methane production, expressed on the basis of digested DM, did not differ between AFEX-treated and untreated wheat straw. However, Griffith et al. (2016) found that CH_4 was decreased with AFEX-treated barley straw compared to untreated straw in the RUSITEC. This reduction in CH_4 could have been due to an increase in propionate and a decrease in the molar proportions of acetate and butyrate. Propionate acts as alternative hydrogen sink in the rumen diverting hydrogen away from the reduction of CO_2 to CH_4 , while the production of butyrate and acetate promotes methanogenesis (Moss et al., 2000). However, in our study, the rapid fermentation of AFEX-treated straw resulted in higher propionate production with no effect on CH_4 production, possibly because there was more propionate produced from the concentrate portion of the diet with untreated wheat straw.

Decreased pH in the fermenters fed AFEX-treated straw also corresponded with the increase in total VFA production. Our results were in agreement with Griffith et al. (2016), as the increase in DM and fiber disappearance with AFEX did correspond to an increase in total VFA production. Similarly to our results, Eun et al. (2006) observed an in vitro decrease in the molar proportion of acetate and an increase in the molar proportion of propionate, resulting in a corresponding decrease in the acetate:propionate, with ammoniated rice straw. Compared to untreated straw, the increase in the molar proportion of propionate and a decrease in acetate with AFEX are in accordance with lower NDF concentration. Ribeiro et al. (2018) treated barley straw with different types of fibrolytic enzymes at 2 doses (100 and 500 μg of protein/g of substrate DM) and found that this generally increased in vitro DM and NDF disappearance and tended to increase ADF disappearance with no effect of dose. Addition of exogenous fibrolytic enzymes did not affect pH and this is in agreement with the reports of Mohamed et al. (2017) who tested addition of fibrolytic enzymes to a total mixed ration (TMR) in a RUSITEC. In the current study, exogenous enzymes tended to increase $\text{NH}_3\text{-N}$ production, possibly due to improved N utilization supporting microbial growth. Although combining enzymes with AFEX-treated straw improved DM and NDF disappearance, there was no effect on total VFA production or molar proportion of acetate and propionate. However, molar proportions of butyrate, isobutyrate, and isovalerate were increased when fibrolytic enzymes were applied to AFEX-treated straw, indicating a shift in the molar proportions of VFA produced. In the

RUSITEC, Giraldo et al. (2007) reported that daily production of butyrate and isovalerate increased when high forage diets were treated with exogenous cellulases. Similar results were also observed by Wang et al. (2012) where exogenous fibrolytic enzymes increased the in vitro NDF digestibility of barley straw and alfalfa hay without affecting VFA production or profiles. Eun et al. (2006) reported that although total VFA production was increased in ammoniated rice straw, further addition of enzymes did not affect total VFA production. In the RUSITEC, Mohamed et al. (2017) reported that digestibility of ADF and NDF improved when exogenous fibrolytic enzymes were added to a TMR consisting of 55% concentrate, 22.5% corn silage, and 22.5% alfalfa hay on a DM basis, but there was no effect on total VFA production or profiles.

Enzyme Activity

Addition of enzymes to AFEX-treated wheat straw increased endoglucanase activity, whereas no effect was observed for untreated straw. The presence of hemicellulose and its associated xylan backbone may have promoted the growth of xylanolytic bacteria. The AFEX treatment reduced the hemicellulose component, as evidenced by the lower NDF concentration. However, untreated straw had higher concentration of hemicellulose (xylan) and this could explain the increased xylanase activity in the feed particle-associated bacterial fraction with untreated straw compared to AFEX-treated straw. Also, the roles of protozoa and fungi in the cellulolytic activity are important (Lee et al., 2000), as rumen protozoa account for up to 30% of the fibrolytic activity in the rumen and fungi play a key role in the degradation of the most recalcitrant plant cell walls (Takenaka et al., 2004; Kamra and Singh, 2017). The lower number of protozoa with AFEX-treated straw may also have contributed to the decreased xylanase activity. Endoglucanase activity in the feed particle-associated fraction increased with the addition of enzymes to AFEX-treated straw, supporting the hypothesis that these fibrolytic enzymes may promote a shift in the microbial population colonizing feed particles.

Microbial Populations

The absolute and relative quantities of methanogens, protozoa, fungi, and *F. succinogenes* were lower with AFEX-treated straw as compared to untreated straw. This suggests that these microorganisms played less of a role in the digestion of this

more readily digestible substrate as compared to untreated straw. In fact, more of the carbohydrates were fermented, increasing the amount of CH₄ produced per day, but with fewer methanogens. This observation suggests that methanogens in fermenters containing AFEX-treated straw were more metabolically active than those in untreated straw.

The most abundant fibrolytic bacterial species in this study was *F. succinogenes* which is consistent with other studies (Koike and Kobayashi, 2009; Zhang et al., 2017). There were positive correlations between the relative quantities of methanogens and *F. succinogenes* and *R. flavefaciens*. The presence of methanogens, particularly *Methanobrevibacter smithii*, can confer a fermentative advantage when co-cultured with *F. succinogenes* or *R. flavefaciens* due to cross-feeding (Rychlik and May, 2000).

Both the quantities of fungi and protozoa were low regardless of treatment, suggesting that neither group thrives in the RUSITEC. Fungi are usually present at 10³–10⁶ zoospores and protozoa at 10⁴–10⁶/mL of rumen fluid (Karri et al., 2016; Wang et al., 2017). In this study, the quantity of fungi in the AFEX-treated straw was below the detectable limit (<200 gene copies) while it was an average of 1.87 × 10³ gene copies with untreated straw. Fewer fungi were expected in the AFEX-treated straw given easier access to rapidly fermentable sugars through the breakdown of the cell wall constituents (Grenet et al., 1989) and competition for those nutrients with faster growing bacteria. The quantity of protozoa was similarly low in this study with an average of 1.2 × 10⁴ gene copies/mL in the AFEX-treated straw and 3.7 × 10⁴ gene copies/mL in the untreated straw. The decline of protozoa with AFEX-treated straw in the absence of a decline in methane production suggests that most of the CH₄ with AFEX-treated straw was being produced by fluid or feed particle-associated methanogens. Copy numbers associated with *S. ruminantium* increased in AFEX-treated rice straw and as this bacterium is a major propionate producer, this may partially account for the higher levels of propionate production as a result of AFEX. *Selenomonas* is also a potent fermenter of mono- and disaccharides (Prins, 1971) and the growth of this bacterium was likely enhanced as a result of the increase in NFC with AFEX.

CONCLUSIONS

Ammonia fiber expansion increased in vitro ruminal degradation of wheat straw and microbial protein synthesis, and improved rumen

fermentation through increased production of propionate. Adding exogenous fibrolytic enzymes also improved the feed degradation. The combination of AFEX-treated straw and exogenous fibrolytic enzymes synergistically improved in vitro ruminal degradability of wheat straw. Future work should include a more comprehensive investigation of the rumen microbiome (including archaea, bacteria, fungi, and protozoa) to determine the overall impact of both AFEX and the addition of fibrolytic enzymes on the rumen microbial ecosystem. Further in vivo studies are required to examine the effect of enzymes combined with AFEX-treated crop residues to more accurately predict the optimal ruminal responses that will promote this synergistic effect.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

LITERATURE CITED

- Adesogan, A. T., S.-C. Kim, K. G. Arriola, D. B. Dean, and C. R. Staples. 2007. Strategic addition of dietary fibrolytic enzymes for improved performance of lactating dairy cows. In: *Proceedings of the 18th Florida Ruminant Nutrition Symposium*, University of Florida, Gainesville. p. 92–110.
- Adesogan, A. T., Z. X. Ma, J. J. Romero, and K. G. Arriola. 2014. Ruminant nutrition symposium: improving cell wall digestion and animal performance with fibrolytic enzymes. *J. Anim. Sci.* 92:1317–1330. doi:10.2527/jas.2013-7273
- Association of Official Analytical Chemists (AOAC). 1995. *Official methods of analysis*. 16th ed. Association of Official Analytical Chemists, Washington, DC.
- Azzaz, H. H., A. M. Kholif, H. A. Murad, M. A. Hanfy, and M. H. Abdel Gawad. 2012. Utilization of cellulolytic enzymes to improve the nutritive value of banana wastes and performance of lactating goats. *Asian J. Anim. Vet. Advances* 7:664–673. doi:10.3923/ajava.2012.664.673
- Badhan, A., G. O. Ribeiro, Jr., D. R. Jones, Y. Wang, D. W. Abbott, M. Di Falco, A. Tsang, and T. A. McAllister. 2018. Identification of novel enzymes to enhance the ruminal digestion of barley straw. *Bioresour. Technol.* 260:76–84. doi:10.1016/j.biortech.2018.03.086.
- Baloyi, T. F. 2008. Effects of exogenous fibrolytic enzymes on in vitro fermentation kinetics of forage and mixed feed substrates. MSc thesis, Stellenbosch University, Stellenbosch, South Africa.
- Bals, B., H. Murnen, M. Allen, and B. Dale. 2010. Ammonia fiber expansion (AFEX) treatment of eleven different forages: improvements to fiber digestibility in vitro. *Anim. Feed Sci. Technol.* 155:147–155. doi:10.1016/j.anifeedsci.2009.11.004
- Blümmel, M., F. Teymouri, J. Moore, C. Nielson, J. Videto, P. Kodukula, S. Pothu, R. Devulapalli, and P. Varijakshapanicker. 2018. Ammonia fiber expansion (AFEX) as spin off technology from 2nd generation biofuel for upgrading cereal straws and stovers for livestock feed. *Anim. Feed Sci. Technol.* 236:178–186. doi:10.1016/j.anifeedsci.2017.12.016
- Buranov, A. U., and G. Mazza. 2008. Lignin in straw of herbaceous crops. *Ind. Crops Prod.* 28:237–259. doi:10.1016/j.indcrop.2008.03.008
- Campbell, T. J., F. Teymouri, B. Bals, J. Glassbrook, C. D. Nielson, and J. Videto. 2013. A packed bed ammonia fiber expansion reactor system for pretreatment of agricultural residues at regional depots. *Biofuels* 4:23–34. doi:10.4155/bfs.12.71
- Canadian Council on Animal Care (CCAC). 2009. CCAC guidelines on: the care and use of farm animals in research, teaching and testing. Can. Counc. Anim. Care, Ottawa, ON, Canada. <http://www.ccac.ca>.
- Chesson, A. 1988. Lignin-polysaccharide complexes of the plant cell wall and their effect on microbial degradation in the rumen. *Anim. Feed Sci. Technol.* 21:219–228. doi:10.1016/0377-8401(88)90104-6
- Chundawat, S. P., R. Vismeh, L. N. Sharma, J. F. Humpala, L. da Costa Sousa, C. K. Chambliss, A. D. Jones, V. Balan, and B. E. Dale. 2010. Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. *Bioresour. Technol.* 101:8429–8438. doi:10.1016/j.biortech.2010.06.027.
- Czerkawski, J. W., and G. Breckenridge. 1977. Design and development of a long term rumen simulation technique (RUSITEC). *Br. J. Nutr.* 38:371–384. doi:10.1079/BJN19770102
- Czerkawski, J. W., and K.-J. Cheng. 1988. Compartmentation in the rumen. In: P. N. Hobson, editor, *The rumen microbial ecosystem*. Elsevier Science Publishing, New York. p. 361–385.
- Dale, B. E., C. K. Leong, T. K. Pham, V. M. Esquivel, I. Rios, and V. M. Latimer. 1996. Hydrolysis of lignocellulosics at low enzyme levels: application of the AFEX process. *Bioresour. Technol.* 56:111–116. doi:10.1016/0960-8524(95)00183-2
- Eun, J. S., and K. A. Beauchemin. 2007. Enhancing in vitro degradation of alfalfa hay and corn silage using feed enzymes. *J. Dairy Sci.* 90:2839–2851. doi:10.3168/jds.2006-820
- Eun, J.-S., K. A. Beauchemin, S.-H. Hong, and M. W. Bauer. 2006. Exogenous enzymes added to untreated or ammoniated rice straw: effects on in vitro fermentation characteristics and degradability. *Anim. Feed Sci. Technol.* 131:87–102. doi:10.1016/j.anifeedsci.2006.01.026
- Fahey, G. C., L. D. Bourquin, E. C. Titgemeyer, and D. G. Atwell. 1993. Postharvest treatment of fibrous feedstuffs to improve their nutritive value. In: H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, editors, *Forage cell wall structure and digestibility*. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, WI. p. 715–766.
- Food and Agriculture Organization (FAO). *Food and Agriculture Organization (FAO) Year Book, Production 2002: FAO Statistics Series*. 176: Vol. 56. 2004. Food & Agriculture Organization of the United Nations, Rome, Italy.
- Gama, R., J. S. Van Dyk, and B. I. Pletschke. 2015. Optimisation of enzymatic hydrolysis of apple pomace for production of biofuel and biorefinery chemicals using commercial enzymes. *3 Biotech* 5:1075–1087. doi:10.1007/s13205-015-0312-7

- Giraldo, L. A., M. J. Ranilla, M. L. Tejido, and M. D. Carro. 2004. Effects of enzyme application method on the in vitro rumen fermentation of tropical forages. *J. Anim. Feed Sci.* 13:63–66. doi:10.22358/jafs/73739/2004
- Giraldo, L. A., M. J. Ranilla, M. L. Tejido, and M. D. Carro. 2007. Influence of exogenous fibrolytic enzymes and fumarate on methane production, microbial growth and fermentation in RUSITEC fermenters. *Br. J. Nutr.* 98:753–761. doi:10.1017/S0007114507744446
- Graham, H., and P. Åman. 1984. A comparison between degradation in vitro and in sacco of constituents of untreated and ammonia treated barley straw. *Anim. Feed Sci. Technol.* 10:199–211. doi:10.1016/0377-8401(84)90009-9
- Grenet, E., A. Breton, P. Barry, and G. Fonty. 1989. Rumen anaerobic fungi and plant substrate colonization as affected by diet composition. *Anim. Feed Sci. Technol.* 26:55–70. doi:10.1016/0377-8401(89)90006-0
- Griffith, C. L., G. O. Ribeiro, Jr., M. Oba, T. A. McAllister, and K. A. Beauchemin. 2016. Fermentation of ammonia fiber expansion treated and untreated barley straw in a rumen simulation technique using rumen inoculum from cattle with slow versus fast rate of fiber disappearance. *Front. Microbiol.* 7:1839. doi:10.3389/fmicb.2016.01839
- Hall, M., P. Bansal, J. H. Lee, M. J. Realff, and A. S. Bommaris. 2010. Cellulose crystallinity—a key predictor of the enzymatic hydrolysis rate. *Febs J.* 277:1571–1582. doi:10.1111/j.1742-4658.2010.07585.x
- Hatfield, R. D., J. Ralph, and J. H. Grabber. 1999. Cell wall structural foundations: molecular basis for improving forage digestibility. *Crop Sci.* 39:27–37. doi:10.2135/cropsci1999.0011183X003900010005x
- Kamra, D. N., and B. Singh. 2017. Anaerobic gut fungi. In: T. Satyanarayana, S. K. Deshmukh, B. N. Johri, editors, *Developments in fungal biology and applied mycology*. Springer, Singapore. p. 125–134. doi:10.1007/978-981-10-4768-8
- Karri, S., M. B. Vadhela, M. Gutta, S. K. Onteru, and V. A. K. B. Gundi. 2016. Role of fiber and non fibrolytic bacteria in feed digestion in bovine rumen—an overview. *Int. J. Pharm. Biol. Sci.* 6:207–211.
- Kerley, M. S., G. C. Fahey, Jr., L. L. Berger, J. M. Gould, and F. Lee Baker. 1985. Alkaline hydrogen peroxide treatment unlocks energy in agricultural by-products. *Science* 230:820–822. doi:10.1126/science.230.4727.820
- Koike, S., and Y. Kobayashi. 2009. Fibrolytic rumen bacteria: their ecology and functions. *Asian-Australas. J. Anim. Sci.* 22:131–138. doi:10.5713/ajas.2009.r.01
- Lee, S. S., J. K. Ha, and K. Cheng. 2000. Relative contributions of bacteria, protozoa, and fungi to in vitro degradation of orchard grass cell walls and their interactions. *Appl. Environ. Microbiol.* 66:3807–3813. doi:10.1128/aem.66.9.3807-3813.2000
- McDougall, E. I. 1948. The composition and output of sheep's saliva. *Biochem. J.* 43:99–109.
- Mendoza, G. D., O. Loera-Corral, F. X. Plata-Pérez, P. A. Hernández-García, and M. Ramírez-Mella. 2014. Considerations on the use of exogenous fibrolytic enzymes to improve forage utilization. *ScientificWorldJournal.* 2014:247437. doi:10.1155/2014/247437
- Menke, K. H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor in vitro. *J. Agric. Sci. (Camb.)* 93:217–222. doi:10.1017/S0021859600086305
- Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31:426–428. doi:10.1021/ac60147a030
- Mohamed, M. A. E., C. Yangchun, B. M. Bodinga, Z. Lixin, Y. Zekun, L. Lihui, J. Yao, R. N. Soomro, I. H. R. Abbasi, and L. Wen. 2017. Effect of exogenous fibrolytic enzymes on ruminal fermentation and gas production by RUSITEC, in vitro abomasum and ileum digestibility. *Int. J. Pharmacol.* 13:1020–1028. doi:10.3923/ijp.2017.1020.1028
- Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* 96:673–686. doi:10.1016/j.biortech.2004.06.025
- Moss, A., J.-P. Jouany, and J. Newbold. 2000. Methane production by ruminants: its contribution to global warming. *Ann. Zootech.* 49:231–253. doi:10.1051/animres:2000119
- Muirhead, S. 1996. Direct fed microbial, enzyme and forage additive compendium. 3rd ed. The Miller 20 Publishing Company, Minnetonka, MN.
- National Academies of Sciences, Engineering, and Medicine (NASEM). 2016. Nutrient requirements of beef cattle. 8th ed. Natl. Acad. Press, Washington, DC.
- Nishimura, H., A. Kamiya, T. Nagata, M. Katahira, and T. Watanabe. 2018. Direct evidence for α ether linkage between lignin and carbohydrates in wood cell walls. *Sci. Rep.* 8:6538. doi:10.1038/s41598-018-24328-9.
- Nsereko, V. L., K. A. Beauchemin, D. P. Morgavi, L. M. Rode, A. F. Furtado, T. A. McAllister, A. D. Iwaasa, W. Z. Yang, and Y. Wang. 2002. Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. *Can. J. Microbiol.* 48:14–20.
- Nsereko, V. L., D. P. Morgavi, L. M. Rode, K. A. Beauchemin, and T. A. McAllister. 2000. Effects of fungal enzyme preparations on hydrolysis and subsequent degradation of alfalfa hay fiber by mixed rumen microorganisms in vitro. *Anim. Feed Sci. Technol.* 88:153–170. doi:10.1016/S0377-8401(00)00225-X
- O'Sullivan, A. C. 1997. Cellulose: the structure slowly unravels. *Cellulose* 4:173–207. doi:10.1023/a:1018431705579
- Oss, D. B., G. O. Ribeiro, Jr., M. I. Marcondes, W. Yang, K. A. Beauchemin, R. J. Forster, and T. A. McAllister. 2016. Synergism of cattle and bison inoculum on ruminal fermentation and select bacterial communities in an artificial rumen (RUSITEC) fed a barley straw based diet. *Front. Microbiol.* 7:2032. doi:10.3389/fmicb.2016.02032
- Peterson, S. J., A. L. Shreck, B. L. Nuttelman, C. J. Schneider, D. B. Burken, C. N. Macken, W. A. Griffin, J. L. Harding, G. E. Erickson, T. J. Klopfenstein, and J. C. MacDonald. 2015. Effects of alkaline treatment and pelleting of crop residues on performance of growing calves. *Prof. Anim. Sci.* 31:552–558. doi:10.15232/pas.2015-01439
- Prins, R. A. 1971. Isolation, culture, and fermentation characteristics of *Selenomonas ruminantium* var. bryantivar. n. from the rumen of sheep. *J. Bacteriol.* 105:820–825.
- R Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.

- Rhine, E. D., G. K. Sims, R. L. Mulvaney, and E. J. Pratt. 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. *Soil Sci. Soc. Am. J.* 62:473–480. doi:10.2136/sssaj1998.03615995006200020026x
- Ribeiro Jr, G. O., A. Badhan, H. Jiangli, K. A. Beauchemin, W. Yang, Y. Wang, A. Tsang, and T. A. McAllister. 2018. New recombinant fibrolytic enzymes for improved in vitro ruminal fiber degradability of barley straw. *J. Anim. Sci.* 9:3928–3942. doi:10.1093/jas/sky251
- Ribeiro Jr., G. O., L. C. Gonçalves, L. G. Pereira, A. V. Chaves, Y. Wang, K. A. Beauchemin, and T. A. McAllister. 2015. Effect of fibrolytic enzymes added to *Andropogon gayanus* grass silage-concentrate diet on rumen fermentation in batch cultures and the artificial rumen (RUSITEC). *Animal* 9:1153–1162. doi:10.1017/S1751731115000221
- Rubin, E. M. 2008. Genomics of cellulosic biofuels. *Nature* 454:841–845. doi:10.1038/nature07190
- Rychlik, J. L., and T. May. 2000. The effect of a methanogen, *Methanobrevibacter smithii*, on the growth rate, organic acid production, and specific ATP activity of three predominant ruminal cellulolytic bacteria. *Curr. Microbiol.* 40:176–180.
- Saleem, A. M., G. O. Ribeiro, Jr, W. Z. Yang, T. Ran, K. A. Beauchemin, E. J. McGeough, K. H. Ominski, E. K. Okine, and T. A. McAllister. 2018. Effect of engineered biocarbon on rumen fermentation, microbial protein synthesis, and methane production in an artificial rumen (RUSITEC) fed a high forage diet. *J. Anim. Sci.* 96:3121–3130. doi:10.1093/jas/sky204.
- Sun, R. C., X. F. Sun, S. Q. Wang, W. Zhu, and X. Y. Wang. 2002. Ester and ether linkages between hydroxycinnamic acids and lignins from wheat, rice, rye, and barley straws, maize stems, and fast-growing poplar wood. *Ind. Crop Prod.* 15(3):179–188. doi:10.1016/S0926-6690(01)00112-1
- Taherzadeh, M. J., and K. Karimi. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *Int. J. Mol. Sci.* 9:1621–1651. doi:10.3390/ijms9091621.
- Takenaka, A., K. Tajima, M. Mitsumori, H. Kajikawa. 2004. Fiber digestion by rumen ciliate protozoa. *Microbes Environ.* 19:203–210. doi:10.1264/jsme2.19.203
- Teymouri, F., L. Laureano-Perez, H. Alizadeh, and B. E. Dale. 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. *Bioresour. Technol.* 96:2014–2018. doi:10.1016/j.biortech.2005.01.016.
- Valdes, K. I., A. Z. M. Salem, S. Lopez, M. U. Alonso, N. Rivero, M. M. Y. Elghandour, I. A. Domínguez, M. G. Ronquillo, and A. E. Kholif. 2015. Influence of exogenous enzymes in presence of *Salix babylonica* extract on digestibility, microbial protein synthesis and performance of lambs fed maize silage. *J. Agric. Sci.* 153:732–742. doi:10.1017/S0021859614000975
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74:3583–3597. doi:10.3168/jds.S0022-0302(91)78551-2
- Wallace, R. J., S. J. Wallace, N. McKain, V. L. Nsereko, and G. F. Hartnell. 2001. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal microorganisms in vitro. *J. Anim. Sci.* 79:1905–1916. doi:10.2527/2001.7971905x.
- Wang, S., K. Giller, M. Kreuzer, S. E. Ulbrich, U. Braun, and A. Schwarm. 2017. Contribution of ruminal fungi, archaea, protozoa, and bacteria to the methane suppression caused by oilseed supplemented diets. *Front. Microbiol.* 8:1864. doi:10.3389/fmicb.2017.01864
- Wang, Y., and T. A. McAllister. 2002. Investigation of exogenous fibrolytic enzyme activity on barley straw using in vitro incubation. *J. Anim. Sci.* 80(Suppl. 1):316.
- Wang, Y., T. A. McAllister, L. M. Rode, K. A. Beauchemin, D. P. Morgavi, V. L. Nsereko, A. D. Iwaasa, and W. Yang. 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the rumen simulation technique (RUSITEC). *Br. J. Nutr.* 85:325–332.
- Wang, Y., J. E. Ramirez-Briebesca, L. J. Yanke, A. Tsang, and T. A. McAllister. 2012. Effect of exogenous fibrolytic enzyme application on the microbial attachment and digestion of barley straw in vitro. *Asian-Australas. J. Anim. Sci.* 25:66–74. doi:10.5713/ajas.2011.11158
- Wang, Y., B. M. Spratling, D. R. ZoBell, R. D. Wiedmeier, and T. A. McAllister. 2004. Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. *J. Anim. Sci.* 82:198–208. doi:10.2527/2004.821198x
- Wang, Z., and L. A. Goonewardene. 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. *Can. J. Anim. Sci.* 84:1–11.
- Wei, T., and V. Simko. 2017. R package “corrplot”: visualization of a correlation matrix (version 0.84). <https://github.com/taiyun/corrplot>.
- Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch, and Y. Y. Lee. 2005. Coordinated development of leading biomass pretreatment technologies. *Bioresour. Technol.* 96:1959–1966. doi:10.1016/j.biortech.2005.01.010
- Yang, B., and C. E. Wyman, 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels Bioprod. Bioref.* 2:26–40. doi:10.1002/bbb.49
- Zhang, L., J. Chung, Q. Jiang, R. Sun, J. Zhang, Y. Zhong, and N. Ren. 2017. Characteristics of rumen microorganisms involved in anaerobic degradation of cellulose at various pH values. *RSC Adv.* 7:40303–40310. doi:10.1039/C7RA06588D