A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions

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ABSTRACT

The objective was to determine if supplementing a dairy cow diet with an exogenous fibrolytic enzyme additive (Econase RDE; AB Vista, Marlborough, Wiltshire, UK) altered fermentation, pH, and microbial populations in the rumen or enteric methane (CH₄) emissions. In a companion study, this enzyme additive improved efficiency of fat-corrected milk production in a dose-dependent manner by up to 11% for early lactation dairy cows. Nine ruminally cannulated, lactating Holstein cows were used in a replicated 3 × 3 Latin square design with 21-d periods. Dietary treatments were 0 (control), 0.5 (low), and 1.0 (high) mL of enzyme/kg of total mixed ration dry matter. Rumen contents were collected on 2 d (d 15 and 19), ruminal pH was measured continuously for 6 d (d 13 to 18) by using an indwelling system, and enteric CH₄ production was measured for 3 d (d 16 to 18) using the sulfur hexafluoride tracer gas technique. The enzyme additive did not alter volatile fatty acids, NH₃, pH, or population densities of total protozoa, bacteria, and methanogens in ruminal fluid. However, population densities of certain bacteria, calculated as copy number of species-specific *16S-rRNA*, were affected by enzyme treatment. Population density of *Ruminobacter amylophilus* was increased and that of *Fibrobacter succinogenes* tended to be increased by the high enzyme treatment. *Selenomonas ruminantium* tended to increase linearly with increasing levels of enzyme in the diet, although its population density was only numerically increased by the high enzyme treatment. *Streptococcus bovis*, however, tended to be decreased by the low enzyme treatment. Increasing the level of enzyme supplement in the diet also linearly increased enteric CH₄ production, even when adjusted for feed intake or milk production (19.3, 20.8, and 21.7 g of CH₄/kg of dry matter intake or 12.9, 13.6, and 15.1 g of CH₄/kg of milk for the control, low, and high

enzyme treatments, respectively). This shift in ruminal bacterial communities and higher CH₄ emissions could imply increased ruminal digestion of feed, which needs to be substantiated in longer term studies.

Key words: exogenous fibrolytic enzyme additive, ruminal bacteria, enteric methane emission, sulfur hexafluoride tracer gas technique

INTRODUCTION

Increases in feed costs continue to fuel interest in improving the production efficiency of dairy cows, and enzyme feed additives with fibrolytic activities offer a possible means of enhancing forage digestion and feed conversion efficiency. A range of effects of using fibrolytic enzymes in ruminant diets has been reported (Beauchemin and Holtshausen, 2011). Some enzyme formulations increased DMI, in vivo fiber digestibility, and milk production of dairy cows, whereas many other studies reported no effects. Responses to enzyme additives vary depending upon the type of animals (energy requirement of the animal and level of feeding), diet composition, enzyme formulation, dose rate, and delivery method of enzyme products (Beauchemin and Holtshausen, 2011).

Recently, some exogenous fibrolytic enzyme products were reported to increase milk production efficiency in early to mid lactation dairy cows (Arriola et al., 2011; Holtshausen et al., 2011). This increased milk production efficiency was a result of a numerical decrease in feed intake with no change in milk yield in cows fed diets supplemented with enzymes. Arriola et al. (2011) related the improvement in feed conversion efficiency to increased total-tract digestibility, although in situ ruminal degradability was not affected. Holtshausen et al. (2011) attributed the improvement in milk production efficiency to an increase in fiber digestibility (based on in vitro assay) of dietary forages. However, ruminal fermentation responses were not measured in that study. Arriola et al. (2011) also reported a similar level of milk production for cows fed an enzyme-treated, high-forage diet (67% diet DM) compared with cows fed an un-

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treated, low-forage diet (52% diet DM). Based on these findings, the use of certain enzyme additives may allow dairy producers to feed higher forage diets or high-fiber byproduct feeds without compromising energy intake and milk production of the cow.

Increased substrate degradability by fibrolytic enzyme additives reported in *in vitro* evaluations (Eun et al., 2007; Eun and Beauchemin, 2007) has often been accompanied by concurrent decreases in acetate:propionate ratios in the incubation fluids. The formation of propionate serves as an H_2 sink in ruminal fermentation, and a greater proportion of propionate and (or) a lower acetate:propionate ratio in ruminal fluid could indicate a lower availability of metabolic H_2 for methanogenesis. Based on this observation, Beauchemin et al. (2008) proposed supplementing ruminant diets with enzyme feed additives as a means of mitigating enteric methane (CH_4) production. Giraldo et al. (2008a) reported that CH_4 production corrected for the amount of OM degraded was decreased when fibrolytic enzymes were added to the substrates during an *in vitro* incubation. In an *in vivo* study (Arriola et al., 2011), a decrease in the calculated enteric CH_4 production was reported for a lactating cow diet (52% dietary forage) supplemented with a fibrolytic enzyme additive. A decreased acetate:propionate ratio was reported in both of the studies.

Hence, this study was conducted to obtain further information on the potential mode of action of the enzyme additive used by Holtshausen et al. (2011), in which milk production efficiency of lactating dairy cows was increased with the use of an exogenous fibrolytic enzyme additive. The objective was to determine the effects of supplementing a dairy cow diet with a fibrolytic enzyme additive on fermentation, pH, and microbial populations in the rumen and enteric CH_4 emissions.

MATERIALS AND METHODS

Experimental Design, Dietary Treatments, and Animal Care

Nine ruminally cannulated, lactating Holstein cows (5 multiparous and 4 primiparous) were used in a replicated 3×3 Latin square design with 21-d periods. Cows were blocked into 3 groups (i.e., squares) on the basis of pre-experimental DIM (means $\pm$ SD: group 1 = 51 ± 8.6 , group 2 = 56 ± 7.5 , and group 3 = 130 ± 36.8). Pre-experimental milk yields for the 3 groups were at (means $\pm$ SD) 41.8 ± 6.0 , 22.7 ± 12.5 , and 46.6 ± 5.4 kg/d for group 1 to 3, respectively. Dietary treatments were 0 (control), 0.5 (low), and 1.0 (high) mL of enzyme/kg of TMR DM.

The basal diet (i.e., the control diet; 48:52 forage to concentrate ratio; Table 1) was formulated to provide adequate ME and MP for a 605-kg cow producing 39 kg/d of milk containing 3.5% fat and 3.3% protein and assuming a DMI of 23 kg/d using the Cornell-Penn-Miner System (CPM Dairy, version 3.0.4a; Cornell University, Ithaca, NY; University of Pennsylvania, Kennett Square, PA; and William H. Miner Agricultural Research Institute, Chazy, NY). The exogenous fibrolytic enzyme additive (Econase RDE; AB Vista, Marlborough, Wiltshire, UK) was obtained from a single batch and contained 722 nmol/ μ L (i.e., nmol of glucose equivalents/min per μ L of the enzyme product) of endoglucanase (EC 3.2.1.4) and 2,604 nmol/ μ L (i.e., nmol of xylose equivalents/min per μ L of the enzyme product) of xylanase (EC 3.2.1.8) activity, with activity determined at 39°C and pH 6.0 using low viscosity carboxymethyl cellulose (catalog no. C-5678; Sigma Chemical Co., St. Louis, MO) and birchwood xylan (catalog no. X-0502; Sigma Chemical Co.) as substrates for endoglucanase and xylanase, respectively. The enzyme additive (i.e., 100 mL of the enzyme additive per 100 kg of TMR DM for the high enzyme treatment) was first diluted in 4 L of water, and the dilution was added to the TMR at the time of mixing, usually within 1 h before feeding. An equal amount of water (4 L/100 kg of TMR DM) was added to the control diet. Experimental diets were fed to cows for *ad libitum* intake once daily at 0800 h. Cows were milked in their stalls twice daily at 0400 and 1400 h.

During each experimental period, d 1 to 14 were used to adapt cows to their assigned experimental diets (the full experimental diets were introduced on d 1 of each period). Rumen contents were collected on d 15 and 19, ruminal pH was measured continuously from d 13 to 18, and enteric CH_4 production was measured from d 16 to 18. This study was conducted separately but at the same time as that reported by Holtshausen et al. (2011) and used the same basal diet and enzyme treatments. Details of animal housing and care were reported in Holtshausen et al. (2011).

Measurements

Daily intakes and refusals of the experimental diets for individual cows were recorded. The refusals for individual cows were sampled twice each week during wk 1 and 2 and from Monday to Friday during wk 3 (the sampling week) of each period and composited by week. Experimental diets and dietary ingredients were sampled twice weekly and composited by week. Weekly refusals, experimental diets, and ingredients were stored at -20°C until analyzed.

Table 1. Ingredients and chemical composition (mean $\pm$ SD) of the basal diet (no enzyme added)

Item	% of diet DM
Ingredient ¹	
Barley silage ²	20.6
Alfalfa silage ³	20.6
Alfalfa hay ⁴	10.8
Ground concentrate mixture ⁵	48.0
Chemical composition	
DM	52.9 $\pm$ 3.62
OM, % of DM	89.5 $\pm$ 0.56
CP, % of DM	20.0 $\pm$ 1.00
NDF, % of DM	33.9 $\pm$ 1.82
ADF, % of DM	23.9 $\pm$ 1.49

¹All ingredients except barley silage, alfalfa silage, and alfalfa hay were provided as part of a ground concentrate mixture.

²Composition (DM basis): 35.3% DM, 15.0% CP, 53.2% NDF, and 34.2% ADF.

³Composition (DM basis): 39.8% DM, 18.9% CP, 37.2% NDF, and 32.2% ADF.

⁴Composition (DM basis): 89.5% DM, 16.8% CP, 47.4% NDF, and 36.6% ADF.

⁵Mixture contained (diet DM basis) 9.52% steam-rolled barley grain, 8.88% steam-rolled corn grain, 6.60% bypass soybean meal (Amino Plus, Ag Processing Inc., Omaha, NE), 6.00% dry ground corn, 5.16% dry ground barley, 4.32% soybean meal, 3.36% corn distillers grain with solubles, 2.52% canola meal, 0.41% limestone, 0.39% sodium bicarbonate, 0.33% salt, 0.19% magnesium oxide, 0.17% dicalcium phosphate, and 0.15% trace mineral premix (added to supply per kg of dietary DM: 65 mg of Mn, 110 mg of Zn, 37 mg of Cu, 1 mg of Co, 0.30 mg of Se, 8,336 IU of vitamin A, 834 IU of vitamin D, and 56 IU of vitamin E).

Milk was sampled from 6 consecutive milkings from d 15 to 18 of each period. Evening and morning milk samples were analyzed separately, and milk composition was calculated accounting for different milk yields between evening and morning milkings. Cows were weighed for 2 consecutive days and BCS assessed (Wildman et al., 1982) at the beginning of each period and at the end of the experiment.

A composite sample of rumen contents was obtained from 5 sites within the rumen (cranial dorsal, cranial ventral, central rumen, caudal dorsal, and caudal ventral locations) at 0, 6, and 12 h after feeding on d 15 and 19. Rumen contents were thoroughly mixed and filtered through 2 layers of polyester monofilament fabric (Pecap 7-255/47, mesh opening 355 μ m; Tetko Inc., Scarborough, ON, Canada). Aliquots of the filtrates were stored on dry ice and then at -80°C until analyzed for bacteria and methanogens or stored at -20°C until analyzed for VFA [by mixing 5 mL of the filtrate with 1 mL of 25% (wt/vol) HPO_3] and NH_3 [by mixing 5 mL of the filtrate with 1 mL of 1% (wt/vol) H_2SO_4]. Another 5 mL of the filtrate was mixed with 10 mL of methyl green-formalin-saline solution for protozoal quantification. Those samples were stored at

20 to 25°C with no exposure to natural or artificial light until analyzed.

Ruminal pH was measured continuously every 30 s for 6 d using the Lethbridge Research Center Ruminal pH Measurement System (LRCpH; Dascor, Escondido, CA) as described by Penner et al. (2006). The system was calibrated at the start and end of the measurement period. Production of enteric CH_4 gas was measured from individual cows for 3 d using the sulfur hexafluoride (SF_6) tracer gas technique with halters and polyvinyl chloride yokes (Johnson et al., 1994) according to McGinn et al. (2009). The permeation tubes (12.5×40 mm) contained $2,562 \text{ mg} \pm 36.9 \text{ SD}$ of ultra-pure SF_6 and were placed in the rumen at least 1 wk before the start of the experiment. The release rates for SF_6 were from 2.9 to 3.3 mg of SF_6/d with a mean release rate of $3.1 \text{ mg} \pm 0.15 \text{ SD } \text{SF}_6/\text{d}$. A halter and a yoke canister were placed on the animal daily immediately before feeding. Background levels of SF_6 and CH_4 were measured by suspending yokes near the ventilation systems. Yoke canisters including the background canisters were removed at 24 h and sampled with a syringe.

Laboratory Analyses

Weekly refusals, experimental diets, and dietary ingredients were measured for DM contents by drying for 48 h at 60°C in a forced air oven. Dry matter contents of ingredients were used to adjust the forage to concentrate ratio of the experimental diets and the amount of enzyme added. Dry matter contents of experimental diets and refusals were used to calculate DMI. The basal diet and dietary ingredients from each period were analyzed for contents of analytical DM, OM, CP, NDF, and ADF (Table 1). Gross energy (GE) contents of the experimental diets were determined using a bomb calorimeter (model E2k; CAL2k, Johannesburg, South Africa). Details of feed sample preparation and analytical methods were reported in Holtshausen et al. (2011).

Concentrations of VFA in ruminal fluid were quantified using gas chromatography (model 5890; Hewlett Packard, Wilmington, DE) and NH_3 determined by the salicylate-nitroprusside-hypochlorite method using a flow injection analyzer (Sims et al., 1995). Concentrations of SF_6 and CH_4 in gas samples from the yoke canisters were analyzed using gas chromatography (model 5890; Agilent Technologies, Santa Clara, CA) with flame-ionization detection for CH_4 and electron capture detection for SF_6 . Standard curves for the gas chromatography were generated throughout the study using 5 gas standards between 18.3 and 299.5 nmol/mol for SF_6 and between 1.6 and 250 $\mu\text{mol/mol}$ for CH_4 . The correlation coefficient exceeded 99.9% for all

curves. Details of gas chromatography conditions for VFA and gas analyses are in Chung et al. (2011).

Ruminal protozoa in filtered ruminal fluid were counted using a counting chamber (Neubauer Improved Bright-Line counting cell, 0.1 mm depth; Hausser Scientific, Horsham, PA) and a light microscope (Ogimoto and Imai, 1981). Duplicate preparations (2 counts per preparation) of each sample were counted (5 out of 9 cells were counted per count) and if either value differed from the average by more than 10%, a third preparation of the sample was counted.

For quantifications of ruminal methanogens, total DNA was extracted from filtered ruminal fluid as described previously (Guan et al., 2008) with modifications. Briefly, ruminal fluid (2 mL) was thawed on ice and added to 4 mL of a wash-buffer solution (10 mmol/L Tris-HCl at pH = 8.0 with 150 mmol/L NaCl). The mixture was vortexed for 30 s and centrifuged at $200 \times g$ for 5 min at 4°C to separate finer feed particles. One milliliter of the supernatant was transferred and subjected to bead beating (Mini Bead-Beater-8; Bio-Spec, Bartlesville, OK) to lyse microbial cells in the supernatant. Total DNA was extracted by mixing the supernatant with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) after physical disruption. Extracted DNA was precipitated in 500 µL of cold ethanol, resuspended in 40 µL of nuclease-free water, and stored at -20°C for further analysis. Quantitative real-time PCR was conducted to quantify archaeal *16S-rRNA* gene using Fast SYBR Green Master Mix kit and StepOnePlus Real-Time PCR System (Applied Biosystems by Life Technologies Corp., Carlsbad, CA). Reaction system, standard curves, and running conditions were reported in Zhou et al. (2009).

For bacteria analysis, extraction of DNA was performed as described previously (Alexander et al., 2011). Briefly, filtered rumen fluid containing finer feed particles and liquid- and solid-associated bacteria was freeze-dried and then ground to a powder using a planetary micro mill (Retsch, Albiheim, Germany). Thirty milligrams of dried rumen fluid was weighed and DNA was extracted using a Qiagen QIAamp DNA Stool Mini Kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions, with the following exception: bacteria were lysed at 95°C for 10 min. DNA was quantified fluorometrically using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Burlington, ON, Canada) with a VersaFluor fluorometer (Bio-Rad, Mississauga, ON, Canada).

Real-time PCR was used to quantify *16S-rRNA* for total bacteria and sequences specific to *Fibrobacter succinogenes*, *Prevotella bryantii*, *Ruminobacter amylophilus*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Selenomonas ruminantium*, and *Streptococcus bovis*.

Primers and annealing temperatures were described previously for *F. succinogenes*, *P. bryantii*, *R. amylophilus*, *R. flavefaciens*, *Sel. ruminantium*, and *Strep. bovis* (Tajima et al., 2001), *R. albus* (Wang et al., 1997), and universal (Nadkarni et al., 2002) *16S-rRNA*. For the PCR, 40 ng of extracted DNA template was added. The PCR cycling conditions, plasmid standards, and reference bacterial strains have been described previously (Wang et al., 2009). Copies of species-specific *16S-rRNA* were normalized to total *16S-rRNA*, and the ratios were used to estimate abundance of bacterial populations.

Calculation and Statistical Analysis

The SF₆ release rates of the permeation tubes did not differ ($P > 0.05$) among treatment groups; thus, the CH₄ data were not covariate-adjusted for the pre-deployment SF₆ release rate. Population densities of protozoa and methanogens were log₁₀ transformed before statistical analysis with the inverse log₁₀ LSM reported. Data for continuous ruminal pH measurement were summarized by day according to Dohme et al. (2008) before statistical analysis. Milk production and DMI were analyzed using the weekly means that were measured during the sampling week of each period.

Data were analyzed using the mixed model procedure (SAS Institute, 1999). Cow was the experimental unit for all variables. The full general linear mixed model included the fixed effects of square, period nested within square, treatment (control, low, or high enzyme), sampling time (day or hour within a day), and the interaction of treatment and sampling time. For milk production and DMI, the fixed effect of sampling time and its related interaction were removed from the full general linear mixed model. For all data, if the interaction of treatment by sampling time was not significant ($P > 0.05$), the fixed effect of sampling time and its related interaction were removed from the full model. Time of sampling was considered a repeated effect (or double repeated effect for ruminal VFA, NH₃, and protozoa) in the model. Cow nested within square was used in the random statement. Denominator degrees of freedom were estimated using the Kenward-Roger option in the model statement. If the effect of enzyme treatment for a variable was significant ($P \leq 0.10$), the treatment LSM was compared with control LSM using the PDIFF option adjusted by the Dunnett method in the lsmeans statement. Time-series covariance structure was modeled using various options and the best time series covariance structure was selected based on the lowest Akaike and Bayesian information criteria. Linear and quadratic dependent variable responses to increasing levels of enzyme in the diet were determined using

Table 2. Ruminal VFA and NH₃ in lactating Holstein cows fed a diet supplemented with a low (0.5 mL/kg of TMR DM) or high (1.0 mL/kg of TMR DM) dose of fibrolytic enzyme additive

Variable	Control	Low enzyme	High enzyme	SEM	Treatment effect, <i>P</i> -value
Total VFA, mM	139	143	141	7.1	0.92
Individual VFA, mol/100 mol					
Acetate (A)	61.6	60.2	61.0	1.15	0.67
Propionate (P)	23.0	23.3	22.7	1.14	0.92
Butyrate	11.4	11.4	12.1	0.38	0.27
Isobutyrate	1.1	1.1	1.1	0.06	0.99
Valerate	1.8	1.8	1.9	0.09	0.25
Isovalerate	1.3	1.3	1.4	0.08	0.57
Caproate	0.35	0.33	0.37	0.028	0.49
A:P ratio	2.7	2.6	2.7	0.16	0.75
NH ₃ , mM	5.8	6.4	5.9	0.33	0.39

orthogonal polynomial contrasts. Data are presented as means $\pm$ standard deviations or LSM $\pm$ standard errors or standard error of the means. Differences were declared significant at $P \leq 0.05$, and a tendency to significance was declared at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

DMI and Milk Yield

Body weights and BCS were similar among treatment groups throughout the experiment, averaging 616 kg (± 14.0 SE) for BW and 2.7 (± 0.07 SE; 1 to 5 scale) for BCS. On the days of CH₄ measurement, DMI was not affected by enzyme, averaging 25.6, 24.4, and 25.5 kg/d (± 1.62 SEM) for the control, low, and high enzyme diet, respectively. Yields of milk or 3.5% FCM that were obtained from the sampling week of each period were also not affected by enzyme, averaging 37.7, 38.2, and 38.0 kg of milk/d (± 1.52 SEM) or 35.7, 36.1, and 36.3 kg of 3.5% FCM/d (± 1.76 SEM) for the control, low, and high enzyme diets, respectively.

Recently, Holtshausen et al. (2011) reported improved production efficiencies of milk or FCM using this exogenous fibrolytic enzyme additive added to the TMR at the same dosages as used in the present study. In Holtshausen et al. (2011), the improvement in production efficiency with enzyme addition, especially at 1.0 mL of enzyme/kg of TMR DM, resulted from a lower level of DMI with no change in milk production. Similar improvements in milk production efficiency were reported by Arriola et al. (2011) for another enzyme additive. Although we used the same basal diet and same batch of enzyme as Holtshausen et al. (2011), in the present study, enzyme addition did not affect milk production efficiency or DMI and milk production. The Latin square design and relatively short periods (3 wk per period) used in our study compared with a complete

randomized block design with 9- to 10-wk periods used by Holtshausen et al. (2011) and Arriola et al. (2011) may be inadequate for evaluating effects of the enzyme treatment on milk production efficiency.

Ruminal VFA, NH₃, and pH

Adding enzyme to the diet did not affect ruminal fluid concentrations of total VFA and NH₃ or molar proportions of individual VFA (Table 2). Concentrations of total VFA and NH₃ and molar proportions of individual VFA in ruminal fluid changed after feeding (hour effect: $P < 0.01$; data not shown). However, no interactions of treatment by sampling time were observed for ruminal VFA or NH₃.

The effects of enzyme feed additives on ruminal fermentation seem to be inconsistent in the literature, even for studies reporting increased digestibility with the addition of fibrolytic enzymes. For example, Arriola et al. (2011) reported an increase in total VFA concentration and a decrease in the acetate:propionate ratio in ruminal fluid. Gado et al. (2009) and Beauchemin et al. (2000) reported an increase in the proportion of acetate in ruminal fluid by fibrolytic enzymes, whereas Beauchemin et al. (1999) and Yang et al. (1999) reported no effect of fibrolytic enzymes on ruminal fermentation.

Dietary addition of enzyme also had no effect on ruminal pH variables (Table 3). The minimum and maximum pH and the daily fluctuations of ruminal pH from the maximum to minimum were similar among treatments. Cows used in this study experienced periods of moderate ruminal acidosis (ruminal pH < 5.8). However, the duration (h/d) and severity (indicated by area between pH 5.8 and the pH profile of the cow) of ruminal acidosis did not differ among treatments averaging 4.2 h/d (± 0.35 SE) for duration and 45.6 pH units $\times$ min/d (± 5.47 SE) for severity.

Table 3. Ruminal pH variables measured continuously for 6 d in lactating Holstein cows fed a diet supplemented with a low (0.5 mL/kg of TMR DM) or high (1.0 mL/kg of TMR DM) dose of fibrolytic enzyme additive

Variable	Control	Low enzyme	High enzyme	SEM	Treatment effect, <i>P</i> -value
Minimum ruminal pH	5.59	5.59	5.58	0.067	0.95
Mean ruminal pH	6.17	6.12	6.13	0.052	0.15
Maximum ruminal pH	6.77	6.66	6.74	0.043	0.11
Range ¹	1.20	1.12	1.16	0.049	0.31
Ruminal pH <5.8					
Duration, h/d	4.2	4.2	4.1	1.33	1.00
Area, pH units × min/d	45.0	40.9	50.8	19.81	0.82
Bouts, no./d	9	11	9	1.8	0.65

¹Range = maximum rumen pH – minimum ruminal pH.

Microbial Population Density in Ruminal Fluid

Population densities of total protozoa, bacteria, and methanogens in ruminal fluid were not affected by enzyme addition (Table 4). However, population densities of certain bacteria, calculated as copy number of species-specific *16S-rRNA* genes, were affected by enzyme treatment. The ruminal fibrolytic bacterium *F. succinogenes* (linear effect: $P = 0.05$) and the non-fibrolytic bacteria *R. amylophilus* (linear effect: $P = 0.01$) and *Sel. ruminantium* (linear effect: $P = 0.10$) increased or tended to increase linearly with increasing levels of enzyme in the diet. Population densities for *F. succinogenes* were 1.7-fold higher (high enzyme vs. control: $P = 0.10$; treatment effect: $P = 0.06$) and *R. amylophilus* were 1.3-fold higher (high enzyme vs.

control: $P = 0.02$; treatment effect: $P = 0.03$) for the high-enzyme treatment compared with the control. The nonfibrolytic bacterium *Strep. bovis*, however, was quadratically affected ($P = 0.03$) by increasing levels of enzyme in the diet, with population density tending ($P = 0.08$) to be lower for the low enzyme treatment versus control. Time relative to feeding affected the population densities of protozoa and bacteria in ruminal fluid (hour effect: $P \leq 0.05$) but this effect was consistent for all treatments. In contrast, time relative to feeding did not influence the population density of ruminal methanogens.

Stimulation of rumen microbial populations has been proposed as a potential mode of action by which exogenous fibrolytic enzymes increase feed digestion and improve animal production (Beauchemin et al., 2003).

Table 4. Population densities of protozoa, bacteria, and methanogens in ruminal fluid from lactating Holstein cows fed a diet supplemented with a low (0.5 mL/kg of TMR DM) or high (1.0 mL/kg of TMR DM) dose of fibrolytic enzyme additive

Variable	Control	Low enzyme	High enzyme	SEM	Treatment effect, <i>P</i> -value
Total protozoa, × 10 ⁵ cell/mL of rumen fluid	3.8	4.0	3.8	0.41	0.98 ¹
Total bacteria, ^{2,3} × 10 ¹⁰ copies of bacterial <i>16S-rRNA</i> /g of DM	7.8	7.9	7.6	0.49	0.92
Specific bacteria, copies of species-specific <i>16S-rRNA</i> / × 10 ⁸					
total bacterial <i>16S-rRNA</i>					
<i>Fibrobacter succinogenes</i> , ⁴ × 10 ⁵	1.9	1.7	3.2†	0.45	0.06
<i>Prevotella bryantii</i> , × 10 ⁴	5.6	7.2	6.4	1.03	0.59
<i>Ruminococcus albus</i> , × 10 ⁴	3.9	4.5	5.4	0.77	0.41
<i>Ruminococcus flavefaciens</i> , × 10 ⁵	1.2	1.2	1.6	0.17	0.23
<i>Ruminobacter amylophilus</i> , ⁴ × 10 ³	2.1	2.2	2.8†	0.21	0.03
<i>Selenomonas ruminantium</i> , ⁵ × 10 ⁴	2.4	2.4	3.2	0.34	0.19
<i>Streptococcus bovis</i> , × 10 ³	1.7	1.3†	1.6	0.46	0.09
Total methanogens, ³ × 10 ⁸ copies of archaeal <i>16S-rRNA</i> /mL of rumen fluid	6.2	12.1	4.6	4.43	0.43 ¹

¹Probability of the treatment effect on total protozoa and methanogens was calculated based on log₁₀ conversion of population densities, which are reported as inverse log₁₀ LSM in the table.

²Population densities for bacteria were measured only in samples taken on d 19 of each period.

³Copies of bacterial or archaeal *16S-rRNA* were quantified by real-time PCR and used to estimate bacterial or methanogenic populations, respectively.

⁴Linear effect with increasing enzyme doses; $P \leq 0.05$.

⁵Tendency to a linear effect with increasing enzyme doses; $0.5 < P \leq 0.10$.

†Treatment LSM differed from control LSM; $P \leq 0.05$. Treatment LSM was compared against the control with the PDIFF command invoked and using the Dunnett adjustment.

‡Treatment LSM tended to differ from control LSM; $0.05 < P \leq 0.10$.

Table 5. Enteric methane (CH₄) emissions from lactating Holstein cows fed a diet supplemented with a low (0.5 mL/kg of TMR DM) or high (1.0 mL/kg of TMR DM) dose of fibrolytic enzyme additive

Variable	Control	Low enzyme	High enzyme	SEM	Treatment effect, <i>P</i> -value
CH ₄ , g/animal per day ¹	471	505	545 [‡]	31.2	0.10
CH ₄ , g/kg of DMI ^{1,2}	19.3	20.8	21.7 [†]	1.12	0.04
CH ₄ , g/kg of MY ^{1,2}	12.9	13.6	15.1 [†]	0.97	0.02
CH ₄ , g/kg of FCM ^{1,2}	13.4	14.3	15.5	0.96	0.13
Percentage GE intake ^{1,2}	6.5	6.9	7.2 [†]	0.38	0.06

¹Linear trend with increasing enzyme doses; $P \leq 0.05$.

²Enteric CH₄ production (g of CH₄/animal per day) expressed relative to DMI determined on the days of CH₄ measurement, yields (kg/d) of milk (MY), or 3.5% FCM produced during the sampling week of each period or gross energy (GE) intake on the days of CH₄ measurement.

[†]Treatment LSM differed from control LSM; $P \leq 0.05$. Treatment LSM was compared against the control with the PDIFF command invoked and using the Dunnett adjustment.

[‡]Treatment LSM tended to differ from control LSM; $0.05 < P \leq 0.10$.

Nsereko et al. (2002) reported increased numbers of total bacteria and cellobiose-utilizing bacteria in dairy cows fed a high concentrate diet (52% diet DM) supplemented with enzymes. Giraldo et al. (2008b) reported an increase in the number of cellulolytic bacteria in sheep fed an enzyme-treated, high forage diet (70% diet DM). Exogenous enzymes were also reported to increase the number of total bacteria (Giraldo et al., 2007) or cellulolytic bacteria (Wang et al., 2001) in vitro using the RUSITEC technique.

Adding exogenous enzymes to the TMR prefeeding appears to increase the availability of feed nutrients to ruminal microbes, as evidenced by increased attachment and colonization of ruminal microbes to feed particles (Wang et al., 2001). A linear increase in population density for *R. amylophilus* and a tendency for a linear increase in population density for *Sel. ruminantium* observed in the current study could be related to a linear increase in *F. succinogenes* with increasing enzyme dosage in the diet. Fermentation products from primary digesters, such as *F. succinogenes*, can attract secondary digesters, such as *R. amylophilus* and *Sel. ruminantium* to attach to and colonize feed particles (Cheng and McAllister, 1997). It is unknown why *Strep. bovis* decreased in the low enzyme treatment but not in the high enzyme treatment. However, the lower population density observed for *Strep. bovis* did not affect ruminal fermentation for the low enzyme treatment compared with control.

Little information exists on the effects of exogenous enzymes on ruminal protozoa and methanogens. Enzyme additives had no effect on protozoa in the present study or in the study by Wang et al. (2001), who evaluated another enzyme additive. Exogenous enzymes increased the number of methanogens in vitro (Dong et al., 1999), but that was not the case in the present study, in which a different enzyme additive was used.

Although the population density of ruminal methanogens was not affected by enzyme, species composition of ruminal methanogens was different between treatments in the present study [data were reported in a companion study by Zhou et al. (2011)].

Enteric Methane Emissions

Increasing the dosage of enzyme supplement increased enteric CH₄ production, even when adjusted for feed intake, milk production, or GE intake (linear effect: $P \leq 0.05$; Table 5). Compared with the control diet, adding a high level of enzyme to the diet increased enteric production of CH₄ (g/head per day) by 16% ($P = 0.07$) or by 12, 17, 16, or 12% after adjusting CH₄ production for DMI ($P = 0.03$), milk yield ($P = 0.01$), 3.5% FCM ($P = 0.09$), or GE intake ($P = 0.04$), respectively.

The observed CH₄ production from the present study may seem to be high. However, after correcting CH₄ production for the amount of milk produced, the observed CH₄ production per unit of milk from cows on the control diet was comparable to that reported in the literature. Holtshausen et al. (2009) reported an intensity of 11.6 g of CH₄/kg of milk for multiparous cows on a control diet using the SF₆ technique, which is similar to the intensity of 11.8 g of CH₄/kg of milk reported for multiparous cows on the control diet in our study. Beauchemin et al. (2009) reported a lower intensity of 11.7 g of CH₄/kg of FCM for primiparous cows fed a control diet compared with an intensity of 13.5 g of CH₄/kg of FCM reported for primiparous cows fed the control diet in our study. The chamber technique was used in Beauchemin et al. (2009). Martin et al. (2008) and Lovett et al. (2005) reported higher CH₄ production per unit milk basis, from 17.4 to 19.6 g of CH₄/kg of milk, than the current study, likely due to a higher

proportion (>65%) of forage in the diets used in their studies. The SF₆ technique was used by both Martin et al. (2008) and Lovett et al. (2005).

A limited body of information describes the effects of enzyme additives on enteric CH₄ emissions from dairy cows. McGinn et al. (2004) reported no effects of a proteolytic enzyme additive on enteric CH₄ emissions. Arriola et al. (2011) fed a fibrolytic enzyme to dairy cows and estimated a reduction in CH₄ production based on ruminal fermentative pattern (a lower acetate to propionate ratio in ruminal fluid by enzyme), but CH₄ was not directly measured. In vitro studies (Eun et al., 2007; Eun and Beauchemin, 2007) reporting improved fiber degradation by fibrolytic enzymes often reported a concurrent decrease in acetate:propionate ratio in the fermentation fluid. Based on this observation, Beauchemin et al. (2008) proposed that dietary enzyme feed additives may help mitigate enteric CH₄ emissions. However, the increased production of enteric CH₄ with increasing levels of enzyme observed in the present study do not support the use of enzymes for enteric CH₄ mitigation and reinforces the need for in vivo evaluation with direct measurement of CH₄ emissions.

An increase in CH₄ production with increasing enzyme dosage indicates that more energy was lost as CH₄ during ruminal fermentation, a finding that may intuitively seem to be at odds with the observed concurrent increase in feed conversion observed by Holtshausen et al. (2011). However, in the study by Holtshausen et al. (2011), increased feed conversion was due to lower DMI that occurred after 5 wk of feeding, and thus, this effect could not be substantiated in a short-term study. It is possible that the increase in feed conversion was due to a change in microbial communities, as reported in the present study. Our results indicate that dietary supplementation with exogenous enzymes caused shifts in ruminal bacterial communities, without an increase in the overall densities of protozoa, bacteria, or methanogens or a change in VFA or pH profile in the rumen, although changes in methanogenic species occurred (Zhou et al., 2011). However, the long-term effect of this fibrolytic enzyme on ruminal variables and enteric CH₄ production still need to be evaluated.

CONCLUSIONS

Addition of an exogenous fibrolytic enzyme additive to the diet of lactating Holstein cows did not alter ruminal fermentation or rumen pH. However, increasing the level of enzyme additive in the diet linearly increased population densities of certain bacterial species and enteric CH₄ production, even when adjusted for changes in feed intake or milk production. More research using

longer term studies is needed for this exogenous fibrolytic enzyme additive to establish whether the observed shift in ruminal bacterial communities improves feed conversion efficiency of dairy cows.

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