Livestock Science ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Use of fibrolytic enzymes additives to enhance *in vitro* ruminal fermentation of corn silage

N. Phakachoed [a], W. Suksombat [a], D. Colombatto [b,c], K.A. Beauchemin [d,*]

[a] *School of Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[b] *Facultad de Agronomía, Universidad de Buenos Aires, C1417DSQ Buenos Aires, Argentina*
[c] *Consejo Nacional de Ciencia y Tecnología (CONICET), Argentina*
[d] *Agriculture and Agri-Food Canada, Research Centre, Lethbridge, Alberta, Canada T1J 4B1*

ARTICLE INFO

Article history:
Received 20 March 2013
Received in revised form
4 June 2013
Accepted 9 June 2013

Keywords:
Exogenous fibrolytic enzyme
Corn silage
Disappearance
In vitro batch culture

ABSTRACT

Two experiments were conducted to evaluate the effect of four enzyme additives on ruminal fermentation of corn silage using a 48 h batch culture *in vitro* assay with buffer and ruminal fluid. Experiment 1 (Exp. 1) and Experiment 2 (Exp. 2) were conducted as completely randomized designs each with two runs and four replicates. The enzyme additives (E1, E2, E3, and E4) were commercial products that provided a range in endoglucanase, exoglucanase, and xylanase activities. For both xylanase (birch wood and oat spelt substrate) and endoglucanase (carboxymethylcellulose substrate), the enzyme products (per ml) were ranked E4 > E1 > E2 > E3. In Exp. 1, the four enzymes were added at 0, 2, 4, and 8 μl/g of corn silage dry matter (DM), whereas in Exp. 2 enzymes were added at 0, 0.5, 1, 2, and 4 μl/g DM. Gas production (GP) was measured at 3, 6, 12, 18, 24, and 48 h after incubation. Disappearance of DM (DMD), neutral detergent fiber (NDFD), and acid detergent fiber (ADFD), and volatile fatty acid concentrations (VFA; total and individual molar proportions) were determined after 24 and 48 h. In Exp. 1, E1 and E2 had higher NDFD and ADFD at 24 and 48 h of incubation ($P < 0.001$) compared with E3 and E4. Increasing dose rate increased NDFD and ADFD for all enzymes (except ADFD for E4 at 48 h), with the optimum dose rate dependant on the enzyme additive (dose × enzyme; $P < 0.01$). There were some treatment effects on DMD and total GP at 24 and 48 h, but these responses were not consistent with responses in NDFD and ADFD. Experiment 2 was conducted to confirm the effects and optimum dose rate of each enzyme additive. In Exp. 2, DMD was not affected by enzyme after 24 and 48 h incubation. There were no enzyme × dose interactions for DMD, NDFD, or ADFD after 24 or 48 h of incubation (except for ADFD at 48 h). After 24 h, DMD, NDFD, and ADFD increased linearly with increasing dose ($P < 0.05$); after 48 h DMD increased linearly, whereas NDFD increased quadratically with increasing enzyme dose ($P < 0.05$). The ADFD increased linearly after 48 h for E3 and E4, but after 48 h ADFD increased quadratically for E1 and E2. Total GP was consistently lowest for E4 at both incubation times ($P < 0.05$). There were no enzyme × dose interactions ($P > 0.05$) for any of the fermentation variables at either 24 or 48 h of incubation in Exp. 2. There were differences amongst the additives for total VFA at 24 and 48 h ($P \leq 0.05$); increasing enzyme dose decreased total VFA after 24 h but increased total VFA at 48 h, such that all doses were higher than the control ($P < 0.001$). Overall, the enzyme additives increased NDFD and ADFD of corn silage *in vitro*; however, E1 and E2 were more effective than E3 or E4. Responses to increasing dose of enzyme

Abbreviations: ADF, acid detergent fiber; ADFD, acid detergent fiber disappearance; DM, dry matter; GP, gas production; NDF, neutral detergent fiber; NDFD, neutral detergent fiber disappearance; TGP, total cumulative gas production; VFA, volatile fatty acid

* Corresponding author. Tel.: +1 403 317 2235; fax: +1 403 317 2182.
E-mail address: karen.beauchemin@agr.gc.ca (K.A. Beauchemin).

1871-1413/$ - see front matter
http://dx.doi.org/10.1016/j.livsci.2013.06.020

were generally linear or curvilinear, and the optimum dose rate differed amongst the products evaluated. Evaluation of the enzymes at 24 and 48 h generally led to the same ranking of the additives, and the degradation of NDF and ADF was more useful in differentiating the enzymes compared with DM and total GP.

1. Introduction

Fibrolytic enzyme feed additives have potential to improve fiber digestion and productivity of ruminants. Forages are high in fiber content, which can limit intake and digestibility of feed by ruminants (Jung and Allen, 1995). Rumen microorganisms produce enzymes that hydrolyze fiber; however, the complex cell wall structure and limited residence time of forage in the rumen limit the extent of fiber digestion by ruminants (Wang and McAllister, 2002). Many studies have evaluated the use of fibrolytic enzyme additives to overcome this limitation (as reviewed by Beauchemin et al. (2003)), with most research focused on cellulases and xylanases that degrade cellulose and hemicellulose, respectively, the major constituents of plant cell walls.

Supplemental fibrolytic enzyme additives have been shown to improve *in vitro* fiber digestion and enhance the nutritive value of both low (Yang and Xie, 2010) and high (Eun et al., 2007) quality forages. Eun and Beauchemin (2007) and Eun et al. (2007) used alfalfa and corn silage as substrates and reported that supplemental enzymes increased digestion of dry matter (DM) and fiber when assessed *in vitro*, which was also observed in continuous culture (Colombatto et al., 2003) and *in vivo* (Rode et al., 1999; Yang et al., 2000). In a study that used grass hay:concentrate (600:400 g/kg DM) as the substrate, fibrolytic enzymes increased total bacterial numbers (Giraldo et al., 2007), and cellulolytic bacteria were increased in rumen simulation (*i.e.*, Rusitec) fermenters using barley grain and alfalfa hay as substrates (Wang et al., 2001). *In vivo* studies have also shown positive responses when supplemental fibrolytic enzymes were fed to ruminants (Arriola et al., 2011; Holtshausen et al., 2011). Although some studies have demonstrated positive effects when using fibrolytic enzymes in ruminant feeds, many other studies have shown inconsistent effects, or no effects on *in vivo* digestibility or animal performance (Knowlton et al., 2002; Lewis et al., 1999; Vicini et al., 2003).

It has been suggested that enzyme additives vary in effectiveness depending upon factors such as enzyme activity, type and dose of enzyme, type of diet, enzyme application method, and animal physiological status (Beauchemin et al., 2003). Thus, a major limitation to widespread commercial use of enzyme technology for ruminants is the uncertainty of effectiveness of enzyme products, as well as the variability in response for a given product depending upon the diet and feeding conditions. It is not yet possible to predict the potential effects of feed enzymes from their biochemical characterization alone (Beauchemin et al., 2004). Thus, conducting an *in vitro* bioassay that reflects the conditions of the rumen can be a useful means of identifying ideal enzyme candidates for use in feeding trials (Beauchemin et al., 2004).

Our project focused on corn silage because it is fed to cattle in many parts of the world and has relatively high nutritive value. While some previous feeding studies have evaluated supplemental enzymes using corn silage based diets (*e.g.*, Arriola et al., 2011), it is not clear what enzyme activities and doses are most effective. It is important to establish optimum dose rate of specific enzyme additives because dose rate directly affects the cost:benefit ratio of feeding enzymes to dairy cows to improve forage digestibility.

Therefore, the objective of the present study was to evaluate *in vitro* the effectiveness and optimum dose rate of various enzyme additives for corn silage. A 24 and 48 h *in vitro* batch culture method was used to examine the effects of four commercial enzyme additives on the ruminal disappearance and rumen fermentation profile of corn silage.

2. Materials and methods

Experiment 1 was conducted as a completely randomized design with two runs (batches) and four replicates per run with 16 treatments arranged as a factorial (4 enzyme additives $\times$ 4 doses). Experiment 2 was conducted as a completely randomized design with two runs and four replicates per run with 20 treatments arranged as a factorial (4 enzyme additives $\times$ 5 doses). In both experiments, the runs were conducted on separate days and the same four enzyme additives and the same corn silage substrate were used.

2.1. Substrate and enzyme product

A laboratory standard corn silage (neutral detergent fiber [NDF], 39.82%; acid detergent fiber [ADF], 19.08%; DM basis) was used as the substrate. Four enzyme additives were evaluated: a 75:25 combination of Cellulase Plus and Xylanase Plus (E1; source organism *Trichoderma longibrachiatum*; Dyadic International, Florida, USA); Rovabio Excel LC2 (E2; source organism *Penicillium funiculosum*; Adisseo France SAS, Antony, France); Rovabio Rips (E3; source organism *P. funiculosum*; Adisseo France SAS, Antony, France), and Econase RDE (E4; *T. longibrachiatum*; AB Vista, Marlborough, UK). The same lot of E1 (3.4 mg/g of total mixed ration DM; corn silage and alfalfa hay were the main forages in the diet) increased fiber digestibility and the efficiency of milk production when fed to dairy cows (Arriola et al., 2011). Similarly, the same lot of E4 had been

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used previously in a dairy study (0.5 and 1.0 ml/kg of total mixed ration DM; forages were barley silage, alfalfa silage, and alfalfa hay) in which milk production efficiency (kg of 3.5% fat-corrected milk/kg of DM intake) linearly increased with increasing enzyme addition (Holtshausen et al., 2011). The E2 is used commercially in non-ruminant feeding, while E3 is an experimental product not commercially available. Neither E2 nor E3 has been evaluated *in vitro* or *in vivo* for ruminants.

2.2. In vitro fermentations

2.2.1. Experiment 1

An *in vitro* batch culture assay was conducted with rumen fluid as the inoculum. The substrate (corn silage) was ground through a 1-mm screen. Approximately 0.9 g DM of the ground corn silage was weighed into an acetone washed and preweighed filter bag (F57, Ankom Technology, Macedon, NY). Four replications were prepared for each treatment for each batch culture incubation time. The enzyme products were diluted with water and then added (200 μl) directly onto substrates in the filter bags (before sealing) at four doses of each enzyme: 0 (control), 2, 4 and 8 μl/g of substrate DM. The bag was heat-sealed, and then placed into an empty 125 ml bottle and incubated at room temperature for 3 h. Rumen fluid was collected from three cannulated cows approximately 3 h after the morning feeding, and strained through four layers of cheesecloth into a flask and flushed with oxygen-free CO_2. Rumen fluid was transported in insulated flasks to the laboratory within less than 1 h of collection. Anaerobic buffer medium (60 ml; Goering and Van Soest, 1970) containing tryptone, buffer, macro and micro mineral solution, resazurin and water was adjusted to pH 6.0 using 1 M trans-aconitic acid (Sigma Chemicals, St. Louis, MO) was added to each bottle. The pH of the buffer was chosen to represent the average pH conditions in the rumen of a dairy cow. In addition to buffer, rumen fluid (15 ml) was added to each bottle in a ratio of 1:4 (rumen fluid:anaerobic buffer medium) under continuous flushing with CO_2. The bottles were closed with rubber stoppers and aluminum seal caps immediately after loading and the bottles were incubated at 39 °C on a rotary shaker for 24 and 48 h. Negative controls (rumen fluid plus anaerobic buffer medium) and blanks (filter bags plus anaerobic buffer medium and rumen fluid) were also incubated using four replications for correction of gas production and disappearance, respectively. Head space gas production (GP) resultant of substrate fermentation was measured at 3, 6, 12, 18, 24, and 48 h post incubation. The GP was measured by inserting a 23 gauge (0.6 mm) needle attached to a pressure transducer connected to a visual display. After 24 and 48 h of incubation, four bottles for each treatment were removed from the incubator, gas pressure was measured, and then bottles were placed on cold water to stop the fermentation. Then the filter bags were removed ($n=4$ for each time and treatment) from bottles and washed under a stream of cold water until the water ran clear. The gas pressure was converted to gas volume using the equation reported by Mauricio et al. (1999).

$$\text{Gas volume (ml)} = 0.18 + (3.697 \times \text{gas pressure, psi}) + (0.0824 \times \text{gas pressure}^2\text{, psi})$$

Total cumulative gas production (TGP, ml) at 24 and 48 h was calculated by summing the gas volumes at each previous measurement time. The pH was measured immediately with a pH-meter. A 5 ml sample of fluid was added to 1 ml of 25% meta-phosphoric acid for measurement of volatile fatty acid (VFA) concentrations (Run 1 only). The washed bags were dried at 55 °C for 48 h and DM disappearance (DMD) was determined by the loss of DM from the bags. The contents of the bags were then assayed for NDF and ADF content, and NDF and ADF disappearance were calculated (NDFD and ADFD).

2.2.2. Experiment 2

The aim of Exp. 2 was to confirm the optimum dose rate of each enzyme additive determined in Exp. 1. Thus, the same enzyme products and the same corn silage were used; however, a narrower range of dose rates was applied (0, 0.5, 1.0, 2.0, and 4.0 μl/g of substrate DM). Experimental procedures were the same as described for Exp. 1, except that VFA were determined on samples from both runs.

2.3. Chemical analysis

The NDF and ADF analyses were conducted sequentially using an ANKOM200 Fiber analyzer unit based on the procedure described by Van Soest et al. (1991). Sodium sulfite (10 g/l NDF solution) and heat-stable bacterial amylase (2 ml/l NDF solution) were used in the analysis of NDF. The VFA were quantified using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA) with a capillary column (30 m × 0.32 mm i.d., 1 μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA), and flame ionization detection. The oven temperature was 150 °C (no hold time), which was then increased by 20 °C/min to 210 °C, and held at this temperature for 2 min. The injector temperature was 225 °C, the detector temperature was 250 °C, and the carrier gas was helium.

The enzyme additives were analyzed for their endoglucanase, exoglucanase, xylanase and α-amylase activities, according to procedures recommended by Colombatto and Beauchemin (2003). The substrates used respectively were medium-viscosity carboxymethylcellulose (Catalog no. C-5678), cellulose (Sigmacell Cellulose; Catalog no. S-3504), xylan (oat spelt, Catalog no. X-0627; birch wood, Catalog no. X-0502), and starch (Catalog no. S-3504) with all substrates sourced from Sigma Chemical (St. Louis, MO, USA). The assay conditions were 39 °C and pH 6.0 to reflect the average pH conditions in the rumen of a dairy cow. A 1% diluted substrate solution (1.0 ml) and 0.1 M citrate phosphate buffer (0.9 ml) were added to test tubes in triplicate and allowed to pre-warm in a water bath at 39 °C. The reaction was initiated by adding 0.1 ml of pre-warmed diluted enzyme solution (diluted in buffer). Incubations were allowed to continue for exactly 15, 120, 5, and 10 min for endoglucanase, exoglucanase, xylanase, and α-amylase, respectively. The reaction was terminated by adding 3.0 ml of 3,5-dinitrosalicylic acid solution.

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Substrate blanks (triplicate) were prepared by adding 1 ml of diluted substrate, 0.9 ml of buffer and 0.1 ml of distilled water. Enzyme blanks were prepared by adding 0.1 ml of diluted enzyme, 0.9 ml of buffer and 1.0 ml of distilled water. After termination of the reaction with dinitrosalicylic acid, tubes were capped with marbles and boiled for 5 min in a water bath. To determine enzymatic activity, 200 μl of the reaction contents was transferred in duplicate into a microtiter plate and absorbance was read at 544 nm against glucose or xylose standards (from 0 to 1 mg) processed under identical conditions. Enzyme activities were expressed as μmol of reducing sugar released/min ml^{-1}.

2.4. Statistical analysis

Data analyses were conducted using the mixed model procedure of SAS (SAS Institute Inc., Cary, NC). Data from Exp. 1 and 2 were analyzed separately as a completely randomized design with enzyme additive, dose and their interaction included in the model as fixed effects. Within experiment run was considered a random effect. When the interaction between enzyme and dose was significant ($P < 0.05$), contrasts and orthogonal polynomial contrasts were performed to determine linear, quadratic and cubic responses to dose within enzyme. When the main effect of dose was significant ($P < 0.05$), contrasts and orthogonal polynomial contrasts were performed to determine overall linear, quadratic and cubic responses to dose. Significance was declared at $P < 0.05$.

3. Results

3.1. Enzyme activity

All enzyme additives supplied xylanase, endoglucanase and exoglucanase activity, but only E2 and E4 supplied amylase activity (Table 1). For both endoglucanase and xylanase activity, the enzyme products (per ml) were ranked E4 > E1 > E2 > E3, regardless of the xylan substrate used (*i.e.*, oat spelt *versus* birch wood). Thus, E4 was the most concentrated source of xylanase and endoglucanase, while E3 was the least concentrated product. The relationship between xylanase determined using either oat spelt or birch wood was strong (Pearson correlation coefficient = 0.98), but E1, E2 and E3 had higher xylanase activity when oat spelt was the substrate whereas E4 had higher xylanase activity when birch wood was used. Additive E1 had highest exoglucanase activity, while E3 had the least exoglucanase activity.

3.2. Experiment 1

After 24 h incubation, there was no difference ($P > 0.05$) between enzyme additives in terms of their effects on DMD or TGP, but enzyme additives differed ($P < 0.05$) in their effects on NDFD and ADFD (Table 2). After 48 h of incubation, in addition to effects on NDFD and ADFD, the enzyme additives also differed ($P = 0.04$) in their effects on DMD, although TGP remained similar for all additives ($P > 0.05$). At both time points, E1 and E2 had higher NDFD and ADFD than E3 and E4 ($P < 0.05$). Effects of enzymes and dose were more prominent for the fiber fractions than for DM. At both incubation times, the effect of enzyme dose on NDFD and ADFD depended upon the additive (enzyme × dose interactions, $P \leq 0.01$). For E1, the response to dose was linear and quadratic (linear only for ADFD after 48 h), with highest NDFD and ADFD at the highest dose for 24 and 48 h. For E2, response to dose was linear, quadratic and cubic (only linear and quadratic for NDFD after 24 h), such that at both incubation times all doses increased NDFD and ADFD compared to the control ($P < 0.05$), with no differences amongst the levels of enzyme applied ($P > 0.05$). For E3, NDFD and ADFD response to dose was linear and quadratic after 24 h of incubation, and just linear after 48 h. As a result, at both incubation times, all doses increased NDFD compared to the control, with no differentiation amongst the doses, but ADFD was only increased with the 4 and 8 μl/g DM doses compared with control. For E4, NDFD and ADFD response to dose was linear (and cubic for NDFD) after 24 h of incubation, such that all doses increased NDFD compared with control with no differentiation amongst the doses ($P > 0.05$). However, only the highest dose increased ADFD after 24 h of incubation ($P < 0.05$). After 48 h of incubation, the response to dose of E4 for NDFD was quadratic, with the 2 and 4 μl/g DM doses higher ($P < 0.05$) than the control, but the highest dose (8 μl/g DM) similar to the

Table 1
Enzyme activity of the four enzyme additives used.

Product[a]	Enzymatic activity[b]				
	Xylanase		Endoglucanase	Exoglucanase	Amylase
	Oat spelt	Birch wood			
E1	1804 ± 26	1721 ± 21	352 ± 5.9	13.9 ± 0.74	–
E2	1372 ± 70	1172 ± 31	159 ± 5.2	8.9 ± 0.05	0.29
E3	616 ± 51	575 ± 11	59 ± 2.5	3.3 ± 0.10	–
E4	3034 ± 41	3979 ± 10	360 ± 16.3	9.3 ± 0.16	0.37

[a] E1: 75:25 combination of Cellulase Plus and Xylanase Plus (Dyadic International, Florida, USA); E2: Rovabio Excel LC2 (Adisseo France SAS, Antony USA); E3: Rovabio Rips (Adisseo France SAS, Antony, France), and E4: Econase RDE (AB Vista, Marlborough, UK).

[b] Endoglucanase, exoglucanase and amylase activity were expressed as μmoles of glucose released per minute per milliliter enzyme. Xylanase activity was expressed as μmoles of xylose released per minute per milliliter enzyme.

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Table 2
Effect of enzyme (E) and dose (D) on the disappearance (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and total cumulative gas production (TGP) from corn silage after 24 and 48 h of incubation in ruminal fluid (Exp. 1) ($N=8$).

Enzyme[1]	Dose (μl/g DM)	24 h				48 h			
		Disappearance (%)			TGP (ml/g DM)	Disappearance (%)			TGP (ml/g DM)
		DM	NDF	ADF		DM	NDF	ADF	
E1	0	46.9	16.7^{c}	8.9^{c}	79.6	56.6	22.7^{c}	14.5^{c}	107.5
	2	48.7	19.5^{b}	13.0^{b}	84.8	57.0	25.6^{b}	17.4^{b}	119.4
	4	50.3	20.2^{b}	13.4^{b}	86.8	57.1	26.2^{b}	18.1^{b}	121.0
	8	49.4	21.7^{a}	15.3^{a}	87.6	58.7	28.1^{a}	22.0^{a}	126.8
	Contrast		*l, q*	*l, q*			*l, q*	*l*	
E2	0	46.9	16.7^{b}	8.9^{b}	79.6	56.6	22.7^{b}	14.5^{b}	107.5
	2	49.8	20.5^{a}	14.1^{a}	83.0	57.1	27.4^{a}	20.0^{a}	121.4
	4	49.8	21.5^{a}	14.6^{a}	85.3	56.8	27.2^{a}	19.7^{a}	123.7
	8	50.6	21.5^{a}	14.7^{a}	84.7	58.3	27.8^{a}	20.1^{a}	126.1
	Contrast		*l, q*	*l, q, c*			*l, q, c*	*l, q, c*	
E3	0	46.9	16.7^{b}	8.9^{b}	79.6	56.6	22.7^{b}	14.5^{b}	107.5
	2	48.1	18.3^{a}	10.6^{b}	82.5	56.7	24.2^{a}	14.7^{b}	118.3
	4	47.9	19.1^{a}	12.8^{a}	82.2	57.0	24.9^{a}	17.0^{a}	122.0
	8	49.2	18.9^{a}	12.4^{a}	82.9	56.9	25.2^{a}	17.2^{a}	122.9
	Contrast		*l, q*	*l, q*			*l*	*l*	
E4	0	46.9	16.7^{b}	8.9^{b}	79.6	56.6	22.7^{b}	14.5	107.5
	2	48.1	18.9^{a}	10.5^{b}	83.6	56.5	24.6^{a}	15.3	115.7
	4	47.5	18.5^{a}	10.3^{b}	83.8	56.9	24.7^{a}	15.6	119.3
	8	48.9	19.5^{a}	12.4^{a}	84.4	55.8	23.8^{ab}	15.2	121.4
	Contrast		*l, c*	*l*			*q*	*ns*	
Dose	0	46.9^{b}	16.7^{c}	8.9^{c}	79.1^{b}	56.6	22.7^{c}	14.5^{c}	107.5^{c}
	2	48.7^{a}	19.3^{b}	12.1^{b}	83.5^{a}	56.8	25.4^{b}	16.9^{b}	118.7^{b}
	4	48.9^{a}	19.8^{ab}	12.8^{b}	84.5^{a}	57.0	25.7^{ab}	17.6^{b}	121.5^{ab}
	8	49.7^{a}	20.4^{a}	13.7^{a}	85.0^{a}	57.4	26.2^{a}	18.6^{a}	124.3^{a}
	Contrast	*l*	*l, q, c*	*l, q, c*	*l, q*		*l, q, c*	*l, q*	*l, q*
P-value									
Enzyme		0.15	<0.001	<0.001	0.14	0.04	<0.001	<0.001	0.36
Dose		<0.001	<0.001	<0.001	<0.001	0.09	<0.001	<0.001	<0.001
Enzyme × dose		0.84	0.01	0.009	0.97	0.08	<0.001	<0.001	0.99
SEM									
Enzyme		2.08	0.27	0.31	7.43	1.32	0.24	0.41	8.12
Dose		2.08	0.27	0.31	7.43	1.32	0.24	0.41	8.12
Enzyme × dose		2.23	0.55	0.61	7.59	1.38	0.49	0.82	8.52

[a,b,c]Means within a column within enzyme or the main effect of dose having different superscript letters are different at $P<0.05$.
l, *q*, *c*: Within a column, the effect of dose for individual enzyme products or the main effect of dose is linear, quadratic, and cubic, respectively, at $P<0.05$.
[1] Enzymes E1, E2, E3 and E4 are identified in Table 1.

control ($P>0.05$). After 48 h of incubation, ADFD was similar ($P>0.05$) for all doses of E4 compared with the control.

For TGP there was no enzyme × dose interaction ($P\geq0.97$) after either 24 or 48 h of incubation (Table 2). After 24 h, all doses had higher TGP than the control, with similar TGP for 2, 4, and 8 μl/g DM. After 48 h, there was a linear and quadratic response in TGP to dose, with greater TGP with 4 and 8 μl/g DM. All enzyme additives showed a similar pattern of GP rate (ml/h) over the 48 h of incubation; GP rate was highest at the beginning of fermentation with peak GP rate at 3 h (data not shown). The rate of GP was lowest for the control at the peak, and at the end of incubation.

After 24 h, neither enzyme nor dose affected ($P>0.05$) total VFA (Table 3). The effect of dose on molar proportion of acetate depended upon the enzyme additive (enzyme × dose interaction, $P=0.04$). Compared with the control, added enzymes had no effect on acetate proportion for E1, E2, and E3, but for E4, 4 and 8 μl/g DM lowered acetate proportion compared with 0 and 2 μl/g DM. Thus, mean acetate proportions were lower for E4 than for the other enzyme additives. Also after 24 h, molar proportion of propionate was higher ($P<0.05$) for E4 and E2 than E1, with E3 being intermediate. Thus, acetate to propionate ratio ($P<0.05$) was highest for E1, intermediate for E3, followed by E2, and lowest for E4. The effect of dose on acetate to propionate ratio after 24 h was enzyme dependent (enzyme × dose interaction, $P=0.008$); acetate to propionate ratio decreased linearly with dose for E3 and E4, whereas the response for E1 was cubic and there was no response for E2.

After 48 h, enzyme additive affected ($P<0.03$) total VFA, but only tended to affect propionate concentration ($P=0.07$) and acetate to propionate ratio ($P=0.08$) (Table 3). Thus, effects of enzymes on total VFA were more pronounced after

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Table 3
Effect of enzyme additive (E) and dose (D) on culture pH and volatile fatty acid (VFA) concentrations from corn silage after 24 and 48 h of incubation in ruminal fluid (Exp. 1) ($N=4$).

Enzyme[1]	Dose (μl/g DM)	24 h					48 h				
		Total VFA (mM)	Molar proportions[2]			Ac:Pr	Total VFA (mM)	Molar proportions[2]			Ac:Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E1	0	122.8	63.9^{ab}	15.9	12.4	4.01^{ab}	131.3	60.5	17.6	13.5	3.44
	2	128.6	64.1^{a}	15.7	12.3	4.10^{ab}	131.0	59.9	17.9	13.6	3.35
	4	119.7	63.4^{b}	16.1	12.6	3.92^{b}	126.9	60.1	17.8	13.5	3.37
	8	122.9	64.1^{a}	15.8	12.2	4.05^{a}	124.9	59.9	18.0	13.5	3.34
	Contrast		*c*			*c*					
E2	0	122.8	63.9	15.9	12.4	4.01	131.3	60.5	17.6	13.5	3.44
	2	118.5	64.0	16.1	12.2	3.93	126.5	59.8	18.1	13.5	3.31
	4	116.4	63.9	16.1	12.2	3.96	120.4	59.9	18.1	13.5	3.31
	8	117.3	63.7	16.3	12.2	3.95	123.2	59.5	18.1	13.7	3.29
	Contrast		*ns*			*ns*					
E3	0	122.8	63.9	15.9	12.4	4.01^{ab}	131.3	60.5	17.6	13.5	3.44
	2	110.4	64.2	15.9	12.1	4.03^{a}	125.6	60.2	18.0	13.2	3.36
	4	108.4	63.9	16.0	12.2	3.99^{ab}	136.8	59.9	17.9	13.6	3.35
	8	117.2	63.7	16.2	12.2	3.92^{b}	133.3	60.2	17.9	13.4	3.36
	Contrast		*ns*			*l*					
E4	0	122.8	63.9^{a}	15.9	12.4	4.01^{a}	131.3	60.5	17.6	13.5	3.44
	2	113.1	63.9^{a}	16.1	12.2	3.89^{a}	136.0	60.1	17.8	13.5	3.38
	4	110.1	62.8^{b}	16.4	12.6	3.83^{ab}	133.7	60.1	17.6	13.5	3.42
	8	120.1	62.9^{b}	16.5	12.5	3.79^{b}	133.3	60.9	17.5	13.2	3.50
	Contrast		*l*, *c*			*l*					
Dose	0	122.3	63.8^{a}	15.9^{b}	12.4^{a}	4.01^{a}	131.3	60.5	17.6	13.5	3.44
	2	117.6	64.1^{a}	15.9^{b}	12.2^{b}	3.99^{b}	129.8	60.0	17.9	13.5	3.35
	4	113.7	63.7^{a}	16.2^{a}	12.4^{a}	3.93^{ab}	129.4	60.0	17.9	13.5	3.36
	8	119.4	63.6^{b}	16.2^{a}	12.3^{ab}	3.93^{c}	128.7	60.1	17.9	13.7	3.37
	Contrast		*l*, *c*	*l*, *c*	*c*	*l*					
P-value											
Enzyme		0.14	0.001	0.001	0.007	<0.001	0.03	0.34	0.07	0.62	0.08
Dose		0.20	0.003	<0.001	0.02	0.003	0.83	0.30	0.08	0.85	0.11
Enzyme × dose		0.93	0.04	0.14	0.11	0.008	0.40	0.80	0.91	0.73	0.85
SEM											
Enzyme		2.87	0.10	0.06	0.05	0.018	2.03	0.19	0.09	0.08	0.026
Dose		2.87	0.10	0.06	0.05	0.018	2.03	0.19	0.09	0.08	0.026
Enzyme × dose		5.75	0.20	0.11	0.09	0.036	4.05	0.38	0.19	0.15	0.052

[a,b,c]Means within a column within enzyme or the main effect of dose having different superscript letters are different at $P<0.05$.
l, *c*: Within a column, the effect of dose for individual enzyme products or the main effect of dose is linear and cubic, respectively, at $P<0.05$.
ns: Within a column, the effect of dose for an individual enzyme product is not linear, quadratic or cubic, at $P>0.05$.
[1] Enzymes E1, E2, E3 and E4 are identified in Table 1.
[2] Expressed as individual VFA, mol/100 mol; Ac=acetate, Pr=propionate and Bu=butyrate.

48 h than after 24 h, but the opposite was true for molar proportions of VFA. Total VFA were higher ($P<0.05$) for E3 and E4 than for E2, with E1 being intermediate ($P>0.05$). There were no effects ($P>0.05$) of dose on total VFA, molar proportion of individual VFA, or acetate to propionate ratio ($P>0.05$), although there was trend ($P=0.08$) for propionate concentration to increase with increasing dose rate.

3.3. Experiment 2

After 24 and 48 h of incubation, DMD was not affected ($P\geq0.22$) by enzyme, but NDFD and ADFD differed ($P<0.01$) amongst enzymes (Table 4). After 24 h, NDFD was lower for E3 than the other enzymes, and ADFD was lower for E3 and E4 compared with E1 and E2. After 48 h, NDFD was lower for E3 and E4 compared with E2, with E1 being intermediate, and ADFD was lower for E3 and E4 compared with E1 and E2.

After 24 h, all enzyme doses increased ($P<0.05$) NDFD and ADFD linearly, such that the highest dose (4 μl/g DM) differed from the intermediate doses (0.5–2.0 μl/g DM), which all differed from the control dose. After 48 h, NDFD increased ($P<0.001$) quadratically with increasing dose, but for ADFD, the effects of dose depended on the enzyme (enzyme × dose interaction, $P=0.005$). For E1 and E2, ADFD responded linearly and quadratically to dose, whereas E3 and E4 responded linearly to dose.

Total GP after 24 h was highest for E1 and E2, and lowest for E4 ($P<0.05$), with all doses equally increasing TGP compared with the control ($P<0.05$). After 48 h, TGP was higher ($P<0.05$) for E1, E2, and E3 compared with E4, and all doses increased TGP compared with the control

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Table 4
Effect of enzyme additives (E) and dose (D) on the disappearance (%) of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF) and total cumulative gas production (TGP) from corn silage after 24 and 48 h of incubation in ruminal fluid (Exp. 2) ($N=8$).

Enzyme[1]	Dose (μl/g DM)	24 h				48 h			
		Disappearance (%)			TGP ml/g DM	Disappearance (%)			TGP ml/g DM
		DM	NDF	ADF		DM	NDF	ADF	
E1	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{c}	96.2
	0.5	44.5	17.6	9.0	70.7	55.9	23.9	12.9^{c}	101.1
	1.0	45.0	17.6	9.0	74.8	56.5	24.7	15.2^{b}	103.4
	2.0	44.2	18.3	9.5	74.4	55.9	25.0	15.8^{b}	100.7
	4.0	44.2	19.7	11.5	76.5	56.4	26.1	18.0^{a}	102.4
	Contrast							*l, q*	
E2	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{c}	96.2
	0.5	43.1	17.8	8.3	76.0	56.5	24.7	14.8^{b}	101.6
	1.0	42.6	17.7	8.8	77.3	56.2	24.9	15.2^{ab}	100.3
	2.0	44.8	18.3	9.9	74.9	56.3	26.0	16.6^{a}	99.2
	4.0	44.4	19.0	10.6	76.3	56.5	25.8	16.3^{ab}	102.8
	Contrast							*l, q*	
E3	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{c}	96.2
	0.5	44.0	16.9	7.9	74.5	55.6	23.9	13.1^{c}	102.3
	1.0	42.9	17.3	7.6	73.1	55.7	23.6	13.3^{c}	102.2
	2.0	44.5	16.4	7.4	73.3	56.0	24.3	13.9^{bc}	101.5
	4.0	45.0	17.5	9.1	73.0	56.2	25.3	15.0^{b}	97.7
	Contrast							*l*	
E4	0	42.5	16.2	7.0	67.7	55.1	22.9	12.5^{ab}	96.2
	0.5	43.8	17.3	7.2	70.6	55.2	22.7	12.3^{b}	93.0
	1.0	42.2	17.1	7.4	71.0	55.7	23.7	13.1^{ab}	96.2
	2.0	42.7	18.0	8.3	71.1	56.0	24.0	13.8^{a}	96.4
	4.0	43.7	18.7	9.0	71.5	56.0	25.2	13.9^{a}	98.7
	Contrast							*l*	
Dose	0	42.5^{b}	16.2^{c}	7.0^{c}	67.7^{b}	55.1^{b}	22.9^{d}	12.5	96.2^{b}
	0.5	43.8^{a}	17.4^{b}	8.1^{b}	73.0^{a}	55.8^{ab}	23.8^{c}	13.3	99.5^{a}
	1.0	43.2^{ab}	17.5^{b}	8.2^{b}	74.0^{a}	56.0^{a}	24.2^{bc}	14.2	100.6^{a}
	2.0	44.0^{a}	17.7^{b}	8.8^{b}	73.4^{a}	56.0^{a}	24.8^{b}	15.0	99.5^{a}
	4.0	44.3^{a}	18.7^{a}	10.0^{a}	74.3^{a}	56.3^{a}	25.6^{a}	15.8	100.4^{a}
	Contrast	*l*	*l*	*l*	*l, q, c*	*l*	*l, q*	*l, q*	*ns*
P-value									
Enzyme		0.22	0.002	<0.001	<0.001	0.37	0.003	<0.001	0.006
Dose		0.03	<0.001	<0.001	<0.001	0.04	<0.001	<0.001	0.05
Enzyme × dose		0.88	0.48	0.32	0.22	1.00	0.77	0.005	0.55
SEM									
Enzyme		0.85	0.35	0.35	0.70	0.29	0.26	0.25	1.16
Dose		0.95	0.40	0.39	0.78	0.33	0.29	0.28	1.30
Enzyme × dose		1.90	0.79	0.77	1.57	0.66	0.57	0.56	2.60

[a,b,c,d]Means within a column within enzyme or the main effect of dose having different superscript letters are different at $P<0.05$.
l, *q*, *c*: Within a column, the effect of dose for individual enzyme products or the main effects of dose is linear, quadratic, and cubic, respectively, at $P<0.05$.
ns: Within a column, the main effect of dose is not linear, quadratic or cubic, at $P>0.05$.
[1] Enzymes E1, E2, E3 and E4 are identified in Table 1.

dose ($P=0.05$). All 4 enzymes showed a similar pattern of GP rate (ml/h) with the highest rate after 18 h of incubation (data not shown).

There were no enzyme × dose interactions ($P>0.05$) for any of the fermentation variables after either 24 or 48 h of incubation (Table 5). After 24 h, total VFA were higher ($P<0.05$) for E1 than the other enzymes, and there was a linear and cubic response to dose ($P=0.008$). By 48 h, total VFA were higher ($P=0.05$) for E2 compared with E1, with the others being intermediate ($P>0.05$). All doses increased ($P<0.001$) total VFA in a quadratic manner at 48 h. After 24 h, there were no treatment differences ($P>0.05$) for molar proportions of VFA or acetate to propionate ratio, but after 48 h, propionate proportion was lower ($P<0.05$) for E4 compared with E1 and E3 and acetate to propionate ratio was higher for E4 and E2 compared with E3. However, there was no effect of dose rate on molar proportions of acetate or propionate or acetate to propionate ratio after 48 h of incubation.

4. Discussion

The enzyme additives evaluated were commercial products, each with a unique range in endoglucanase,

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Table 5
Effect of enzyme additive (E) and dose (D) on the ruminal pH and volatile fatty acids (VFA) concentrations from corn silage after 24 and 48 h of incubation in ruminal fluid (Exp. 2) ($N=8$).

Enzyme[1]	Dose (μl/g DM)	24 h					48 h				
		Total VFA (mM)	Molar proportions[2]			Ac:Pr	Total VFA (mM)	Molar proportions[2]			Ac:Pr
			Ac	Pr	Bu			Ac	Pr	Bu	
E1	0.5	116.9	61.0	17.5	13.1	3.49	126.8	56.2	20.4	14.5	2.76
	1.0	120.9	61.2	17.7	13.0	3.47	127.7	56.1	20.4	14.5	2.75
	2.0	119.9	61.4	17.6	12.7	3.49	124.3	56.6	20.2	14.3	2.80
	4.0	118.7	61.4	17.5	12.8	3.52	130.0	56.6	20.2	14.2	2.81
E2	0	117.9	60.4	17.4	13.4	3.49	122.2	56.1	20.0	14.7	2.81
	0.5	105.8	61.1	17.5	12.9	3.50	130.2	56.8	20.1	14.1	2.84
	1.0	110.6	61.4	17.5	12.8	3.51	135.5	57.1	19.7	14.2	2.90
	2.0	111.4	60.8	17.8	13.1	3.42	140.8	57.3	20.0	13.9	2.88
	4.0	108.8	60.8	17.9	13.0	3.41	135.4	56.3	20.1	14.5	2.81
E3	0	117.9	60.4	17.4	13.4	3.49	122.2	56.1	20.0	14.7	2.81
	0.5	111.8	60.8	17.8	13.1	3.42	136.5	57.0	20.0	14.2	2.86
	1.0	106.6	60.9	17.6	13.0	3.47	133.3	55.3	20.4	14.8	2.71
	2.0	115.5	61.1	17.6	12.9	3.47	138.0	56.2	20.2	14.4	2.78
	4.0	108.4	61.3	17.6	12.9	3.50	125.0	54.8	20.8	14.9	2.63
E4	0	117.9	60.4	17.4	13.4	3.49	122.2	56.1	20.0	14.7	2.81
	0.5	116.7	61.6	17.4	12.6	3.55	127.1	55.9	19.8	14.7	2.82
	1.0	107.2	61.6	17.5	12.6	3.53	128.7	56.6	19.7	14.4	2.87
	2.0	106.9	60.4	17.8	13.1	3.40	132.9	57.1	19.4	14.2	2.94
	4.0	107.9	60.7	18.0	12.9	3.38	128.1	56.4	19.5	14.7	2.90
Dose	0	117.9^{a}	60.4^{b}	17.4^{b}	13.4^{b}	3.49	122.2^{b}	56.1	20.0	14.7^{a}	2.81
	0.5	112.8^{b}	61.2^{a}	17.6^{a}	12.9^{a}	3.49	130.1^{a}	56.6	20.1	14.4^{bc}	2.82
	1.0	111.3^{b}	61.3^{a}	17.6^{a}	12.8^{a}	3.49	130.4^{a}	56.3	20.0	14.5^{ab}	2.81
	2.0	113.5^{b}	60.9^{ab}	17.7^{a}	12.9^{a}	3.45	134.0^{a}	56.8	20.0	14.2^{c}	2.85
	4.0	111.0^{b}	61.1^{a}	17.7^{a}	12.8^{a}	3.45	129.6^{a}	56.0	20.2	14.6^{ab}	2.79
	Contrast	*l, c*	*c*	*l, c*	*l, q, c*		*l, q*			*q*	
P-value											
Enzyme		<0.001	0.92	0.87	0.76	0.84	0.05	0.02	<0.001	0.04	<0.001
Dose		0.008	0.003	0.001	<0.001	0.15	0.001	0.10	0.80	0.001	0.43
Enzyme × dose		0.07	0.35	0.17	0.86	0.12	0.48	0.07	0.13	0.31	0.08
SEM											
Enzyme		2.10	0.33	0.09	0.22	0.0358	1.77	0.29	0.10	0.17	0.027
Dose		2.35	0.37	0.10	0.24	0.039	1.98	0.33	0.12	0.20	0.030
Enzyme × dose		4.70	0.74	0.21	0.48	0.078	3.95	0.65	0.23	0.39	0.061

[a,b,c]Means within a column for the main effect of dose having different superscript letters are different at $P<0.05$.
l, *c*, *q*: Within a column, the main effect of dose is linear, cubic or quadratic at $P<0.05$.
[1] Enzyme E1, E2, E3 and E4 are identified in Table 1.
[2] Expressed as individual VFA, mol/100 mol; Ac=acetate; Pr=propionate, and Bu=butyrate.

exoglucanase and xylanase activities. Although not assayed in our study, other minor fibrolytic enzymic activities likely also varied amongst these additives. Enzyme additives typically have a wide range of fibrolytic activities depending on the organism used to produce the enzyme, the growth substrate, and the culture conditions employed for enzyme production as reviewed by Beauchemin et al. (2004).

There is a lack of standardization of methodology used to assay enzyme activity of ruminant feed enzymes. As indicated by Colombatto and Beauchemin (2003), the resulting enzymic activity is a function of the conditions of the enzyme assay, particularly the substrate used, temperature, and pH. In our study both oat spelt and birch wood xylan were used in the determination of xylanase. There was a strong correlation between the xylanase activities determined using these two substrates, and thus the enzyme additives ranked similarly using either xylan substrate. However, E1, E2 and E3 had higher xylanase activity when oat spelt was used, whereas E4 had higher xylanase activity when birch wood was used. Birch wood and oat spelt differ in their composition, and hence result in different xylanase activity when used as xylan substrates. The structure of xylan from different sources depends on extraction procedures and degree of substitution of the xylan backbone with other residues (Ghatora et al., 2006). Xylans from grasses and cereals (*e.g.*, oat spelt) contain arabinofuranosyl and glucopyranosyl uronic acid substituents, whereas xylans from hardwoods (*e.g.*, birch wood) contain substantial amounts of glucopyranosyl uronic

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acid and very small amounts of arabinofuranosyl substituents (Kormelink and Voragen, 1993). Thus, oat spelt xylan is usually considered to be a more representative substrate for ruminant feed enzymes. Kung et al. (2002) reported different activity profiles when enzyme additives were assayed at different pH values and suggested that if the additives are to be supplemented at the time of feeding, the most effective additives would have high activity at a pH range reflective of conditions in the rumen. The same rationale can be used for temperature. In our study, the enzyme assays were conducted at pH 6 and 39 °C as suggested by Colombatto and Beauchemin (2003) to reflect the mean ruminal conditions of a typical dairy cow fed a diet containing forage and concentrate.

The present study evaluated several commercially produced fibrolytic enzyme additives for their potential to be used as ruminant feed additives for corn silage based diets. *In vitro* techniques are often used as a bioassay to predict *in vivo* response to exogenous enzymes because animal responses cannot be predicted from enzyme activities alone (Beauchemin et al., 2004). Furthermore, conducting animal feeding studies is very costly, thus it is important to conduct preliminary screening of enzymes to determine their potential for further evaluation. While an *in vitro* assay can be useful for identifying effective enzymes for feeding studies, factors such as scaling up the dose rate from *in vitro* to *in vivo*, method of adding the enzyme to feed, differences in composition of the diet, and animal variability can influence whether effects observed *in vitro* are also observed *in vivo* (Beauchemin et al., 2004). Because the *in vitro* assay was used in our study to recommend enzymes for future use in dairy cow feeding studies, the pH of the buffer used was adjusted to pH 6 to reflect the typical pH in the rumen of dairy cows. The final pH after 48 h of incubation ranged from 5.69 to 6.00 (data not shown). The relatively low pH of the batch culture assay in this study resulted in NDFD and ADFD values at 48 h that would be expected to be lower than had a buffer with higher pH been used in the *in vitro* assay.

To ensure our *in vitro* screening methodology was relevant to *in vivo* results, we used two (E1 and E4) additives that had been used previously in feeding studies with dairy cows where positive results had been reported. However, corn silage was only used in the study by Arriola et al. (2011), who allocated 60 dairy cows in early lactation to high (520 g/kg roughage, including 370 g/kg corn silage) and low (670 g/kg roughage, including 490 g/kg corn silage) concentrate diets with and without enzyme (E1) supplementation (DM basis; 3.4 mg of enzyme/g of ration DM). Milk production efficiency (kg of 3.5% fat-corrected milk/kg of DM intake) increased by 16% for the low concentrate diet, and by 6% for the high concentrate diet. In a companion metabolism using the same treatments, total tract digestibility of DM, crude protein, NDF and ADF were all increased with supplemental enzymes, regardless of level of roughage in the diet. Thus, E1 was considered a positive control in our *in vitro* study.

Although there were some small differences between the results for Exp. 1 and 2, generally Exp. 2 confirmed the results observed in Exp. 1. Based on improvements in NDFD and ADFD in both studies, E1 and E2 were more effective than E3 and E4 after both 24 and 48 h of incubation (Fig. 1). Enzyme E1 increased NDFD respectively by up to 30% and 24% at 24 and 48 h, in Exp. 1 and by up to 22% and 14%, in Exp. 2. Similarly, E2 increased NDFD respectively by up to 29% and 22% at 24 and 48 h, in Exp. 1 and by up to 17% and 14%, in Exp. 2. For all enzymes, improvements in ADFD were greater than for NDFD. For E1, ADFD respectively increased by up to 72% and 52% at 24 and 48 h, in Exp. 1 and by 65% and 44%, in Exp. 2. For E2, ADFD respectively increased by up to 65% and 39% at 24 and 48 h, in Exp. 1 and by 51% and 33%, in Exp. 2. Thus, the increases in NDFD and ADFD were fairly similar for E1 and E2. Given that E1 improved performance of dairy cows fed a diet containing corn silage (Arriola et al., 2011), it is recommended that E2 be further evaluated in dairy cow feeding studies using diets based on corn silage.

Maximum improvements in NDFD for E3 were respectively 13% and 11% after 24 and 48 h, in Exp. 1, and 8% and 10%, in Exp. 2 (Fig. 1). For ADFD these were respectively 39% and 19%, in Exp. 1 and 30% and 20%, in Exp. 2. While positive, these improvements were lower than for E1 and E2, and thus if E3 is to be used as a feed additive for dairy cows, it would need to be priced significantly lower than E1 and E2 such that higher dose rates could be used.

For E4, maximum improvements in NDFD and ADFD were of the same magnitude as observed for E3 (Fig. 1). In Exp. 1, maximum improvement in NDFD for E4 was 17% after 24 h and 5% after 48 h, and in Exp. 2, 15% and 10%, respectively; for ADFD these were 39% after 24 h and 5% after 48 h in Exp. 1 and 29% and 11%, respectively, in Exp. 2. This enzyme additive was used in a study by Holtshausen et al. (2011) in which 60 dairy cows in early lactation were fed diets containing no enzyme, low enzyme (E4; 0.5 ml of enzyme/kg of diet DM), and high enzyme (E4; 1.0 ml of enzyme/kg of diet DM). The diet contained 520 g/kg roughage, including 206 g/kg barley silage, 206 g/kg alfalfa silage and 108 g/kg alfalfa hay (DM basis). Adding enzyme to the diet linearly increased milk production efficiency (kg of 3.5% fat-corrected milk/kg of DM intake) by up to 11%. It is interesting to note that when the authors used E4 in a 24 h *in vitro* batch culture using each of the forages individually, improvements in NDFD and ADFD were only observed for alfalfa hay, and only at a higher dose rate (2 ml of enzyme/kg of forage DM). Given the improvements in NDFD and ADFD observed in our study using corn silage compared to the very minor improvements seen *in vitro* by Holtshausen et al. (2011) for other forages, it is possible that E4 would result in positive effects if used *in vivo* with a diet containing corn silage.

One of the objectives of our study was to determine the dose response of the enzymes to determine optimum dose for each product. Given that the response to enzyme dose differed for the variables measured and the incubation time, optimum dose is somewhat subjective. Thus, we considered optimum dose to be the dose at which NDFD and ADFD were increased compared with the control, with only minor further improvements with higher doses of enzyme. For E1, highest NDFD and ADFD was observed at the highest level for both incubations times in both studies, thus optimum dose was 8 μl/g DM. For E2, the optimum dose was 2 μl/g DM because it increased NDFD

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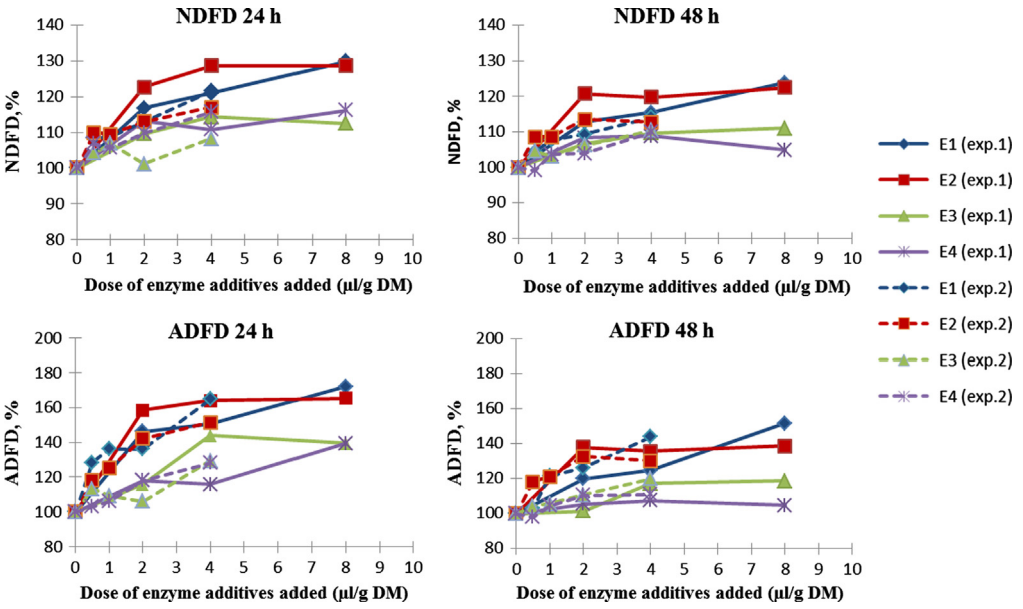

Fig. 1. Increase in neutral detergent fiber disappearance (NDFD) and acid detergent fiber disappearance (ADFD) from corn silage relative to the control after 24 and 48 h of incubation in Exp. 1 and 2. Enzymes E1, E2, E3 and E4 are identified in Table 1.

and ADFD in Exp.1, with only minor further improvements at higher doses, and responses at low dose rates in Exp. 2 were generally linear. For E3, optimum dose rate was 4 µl/g DM, because a further increase in enzyme addition failed to further increase ADFD. For E4, optimum dose rate was 2 µl/g DM. Thus, the effect of enzyme dose on improving fiber digestion differed amongst enzyme products. As each enzyme additive provides a unique array of enzymic activities, differences in the responses among additives, and in optimum dose rate, was anticipated. Similarly, Eun et al. (2007) reported that *in vitro* disappearance of NDF and ADF from alfalfa hay and corn silage were increased by exogenous fibrolytic enzymes, but the response depended upon the enzyme and its dose, with some additives effective for both forages when added at 1.4 mg/g of DM, but others only moderately effective for either forage.

Increasing NDFD and ADFD of corn silage through the addition of enzyme additives would be expected to increase ruminal fiber digestibility and DM intake of dairy cows, through the reduction of physical fill in the rumen. Digestibility of NDF measured *in vitro* or *in situ* has been shown to be a good indicator of the potential of forage to enhance DM intake (Oba and Allen, 1999). Jung et al. (2004) reported that an increase of one percentage unit in *in vitro* NDF digestibility of corn silage resulted in a 0.14 kg/d increase in 3.5% fat-corrected milk yield and a 0.12 kg/d increase in DM intake by dairy cows fed a diet high in corn silage proportion (> 400 g/kg DM). Moreover, Oba and Allen (1999) also reported a positive relationship between forage NDF digestibility (*in vitro* or *in situ*) and milk production and DM intake.

The magnitude of the responses in NDFD and ADFD to increasing dose differed amongst enzyme additives in a manner that could not be explained by activity of xylanase (oat spelt) or endoglucanase (Figs. 2 and 3). In other words, enzyme activity alone could not be used to predict improvement in NDFD or ADFD. One probable reason for this is that enzyme activities are measured on model substrates that do not represent the complexity of plant cell wall material (Beauchemin et al., 2004).

Based on TGP and DMD there was little to no differentiation in the effectiveness of the enzymes at 24 h in both studies, and at 48 h, E1, E2, and E3 were more effective than E4. Thus, degradation of NDF and ADF was more useful in differentiating the enzymes compared with DM and TGP.

Volatile fatty acids are end-products of rumen microbial fermentation and represent the main supply of energy for ruminants. The observed increases in total VFA concentration with added enzymes, and the changes in molar proportions of VFA, were somewhat inconsistent between 24 and 48 h and between Exp. 1 and Exp. 2. The increases in total VFA concentration did not correspond to increases in DMD, NDFD or ADFD. Furthermore, in Exp. 1 at 24 h, E1 had the highest, and E4 had the lowest, acetate to propionate ratio, but these differences were not maintained at 48 h. Those differences in acetate to propionate ratio were not observed at 24 h in Exp. 2, and by 48 h E4 actually had the highest ratio and E1 had the lowest ratio. In comparison, in the *in vivo* study by Arriola et al. (2011), total VFA increased and acetate:propionate ratio decreased with added enzyme (E1). Chung et al. (2012) reported no effect of adding enzyme (E4) to a diet that did not contain corn silage on ruminal fluid concentrations of total VFA or molar proportions of individual VFA. Our data suggest that measuring effects of enzyme on VFA concentrations are not a particularly useful way of screening the potential effects of the enzyme additives *in vivo*.

Our *in vitro* assay focused on both 24 and 48 h of incubation, as it was not clear whether both time periods would produce similar results. Most *in vitro* incubation

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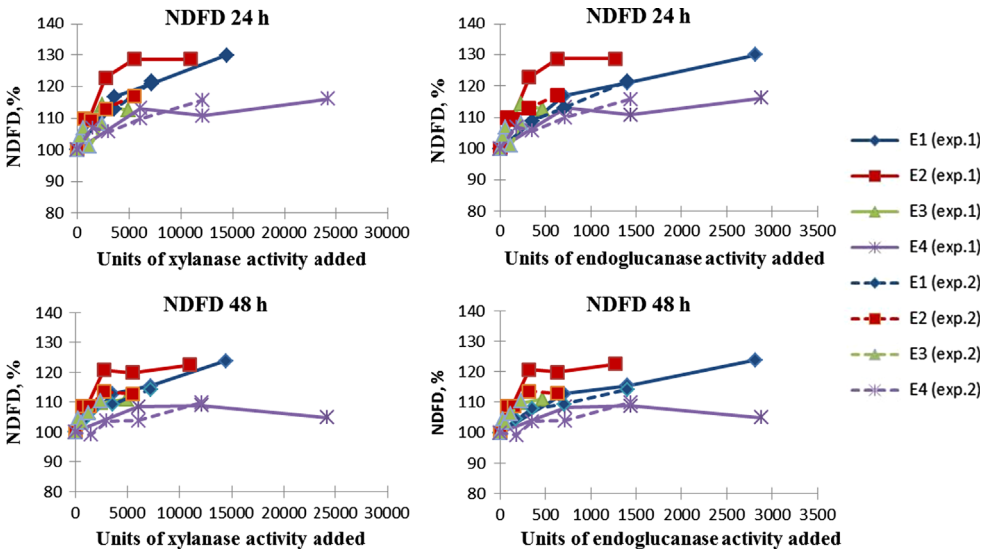

Fig. 2. Added activities of endoglucanase and xylanase (oat spelt) and increase in neutral detergent fiber disappearance (NDFD) from corn silage relative to the control after 24 and 48 h of incubation in Exp. 1 and 2. Enzymes E1, E2, E3 and E4 are identified in Table 1. Endoglucanase activity was expressed as μmoles of glucose released per minute per milliliter enzyme; xylanase activity was expressed as μmoles of xylose released per minute per milliliter enzyme.

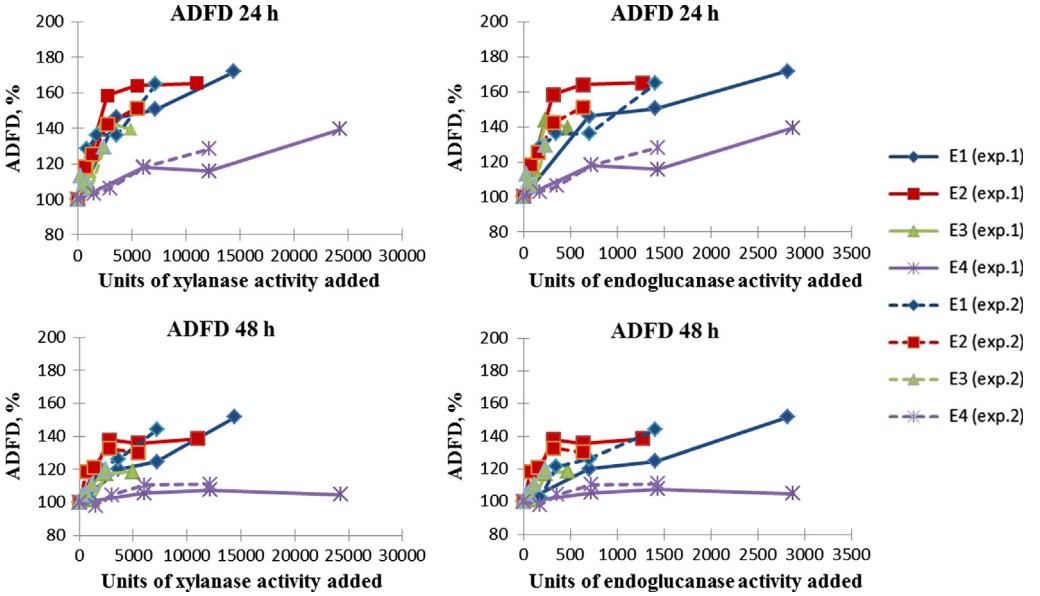

Fig. 3. Added activities of endoglucanase and xylanase (oat spelt) and increase in acid detergent fiber disappearance (ADFD) from corn silage relative to the control after 24 and 48 h of incubation in Exp. 1 and 2. Enzymes E1, E2, E3 and E4 are identified in Table 1. Endoglucanase activity was expressed as μmoles of glucose released per minute per milliliter enzyme; xylanase activity was expressed as μmoles of xylose released per minute per milliliter enzyme.

times used to evaluate fiber degradability range between 24 and 48 h to reflect the mean retention time of forages in the rumen. In a review of the literature, Owens and Goetsch (1986) reported that passage rate of roughage in beef and dairy cattle consuming $>2.25\%$ of body weight averaged 4.5%/h (mean retention time of 22 h), and for cattle consuming diets containing 20–50% concentrate, passage rate of roughage was 3.7%/h (mean retention time of 27 h). The National Research Council (2001) uses a 48 h *in vitro* incubation to predict ruminal NDF digestibility of dairy cows at maintenance. Eun and Beauchemin (2007) focused on 24 h *in vitro* batch culture fermentation and found exogenous enzymes improved *in vitro* disappearance of alfalfa and corn silage. When considering fiber degradation at higher dose rates (Exp. 1), the results were similar at 24 and 48 h, thus only one time point would have been necessary. However, at lower dose rates (Exp. 2) differences amongst enzymes were more pronounced at

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48 h. Furthermore, for ADFD the enzyme dose response depended upon the enzyme. Thus, the 48 h incubation was most useful in terms of defining the dose response and differentiating amongst enzyme products. Overall, in terms of using an *in vitro* assay to evaluate enzymes for further study *in vivo*, we recommend examining the effects on NDFD and ADFD at both 24 and 48 h, to achieve a more comprehensive understanding of the enzyme-forage response.

5. Conclusions

The enzyme additives evaluated in the present study supplied a unique range of endoglucanase, exoglucanase and xylanase activities. All four additives evaluated increased NDFD and ADFD of corn silage *in vitro*, with E1 and E2 being more effective than E3 and E4. Dose response was enzyme dependent for most variables. For E1, maximum response was observed at the highest level (8 μl/g DM). For E2, a dose of 2 μl/g DM increased fiber disappearance, with limited further increase with increasing dose rate. For E3, optimum dose rate was 4 μl/g DM, because a further increase in enzyme addition failed to further increase ADFD. For E4, optimum dose rate was 2 μl/g DM. In general, NDF and ADF were more useful in differentiating the enzymes compared with DM and TGP. Based on the responses observed, further study of these additives for dairy cows fed corn silage diets is recommended.

Conflict of interest statement

None of the authors have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, this work.

Acknowledgments

The authors are grateful to Thailand Research Fund's Royal Golden Jubilee PhD Program for providing financial support to N. Phakachoed. We also thank A. Furtado, B. Farr, D. Vedres, D. Vyas and A. Nanon (Research Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada) for their assistance in sampling and laboratory analyses. The research was funded by the Adisseo France SAS, Antony, France and the enzyme additives were provided by Adisseo France SAS, Antony, France (E2, E3), AB Vista, Marlborough, UK (E4), and Dyadic International, Florida, USA (E1).

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