Animal Nutrition and Feed Technology

Volume 13 **Number 3** **September 2013**

Special Issue

EXOGENOUS ENZYMES IN ANIMAL NUTRITION – BENEFITS AND LIMITATIONS

Guest Editor

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www.anft.org

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PREFACE

Exogenous Enzymes in Animal Nutrition - Benefits and Limitations

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The use of exogenous enzymes in animal nutrition dates back to the mid-1920s, however, nowadays the development of interdisciplinary sciences exploiting molecular methods create new opportunities and deliver new tools to assess effectiveness of their utilization. The proper use of enzymes in animal nutrition allows to obtain maximum benefit from their action not only for the animals, but also for the environment.

The strategies of exogenous enzymes utilization in nutrition of high yielding non-ruminant and ruminant animals are intended to be published in this special issue. Effectiveness of enzymes in animal nutrition depends on (i) type, (ii) source, (iii) level of supplemented enzymes, as well as (iv) the type of diet fed, (v) animal health and (vi) animal productivity. Hence, research focused on the effect of phytase and non-phytase enzyme segments, enzymes combinations, including enzymes produced by genetically modified bacteria, protozoa and fungi, on enteric fermentation, animal health and productivity are desirable.

The current special issue covered the highlighted topics: mode of action of particular enzymes and their combinations, occurrences of synergism and antagonism reactions in relation to enzymes themselves and to dietary ingredients; the impact of enzymes on nutrient utilization including basic nutrients components, antinutritional factors, minerals and, consequently, enteric microbial populations, nutrients digestibility, growth performance; the optimum enzymes dosages as dietary supplements; optimalization of enzyme activity to make enzymes activity more effective from economic and ecological points of view; alternative use of enzymes in mixtures of other feed additives e.g. organic acids; methods of enzymes application.

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The review process for this issue tended to focus on one of the topics mentioned in the objectives above, but all have in common the use of exogenous enzymes and their impacts on rumen fermentation and/or animal welfare and/or animal performance and health as well as performance of non ruminant animals.

Guest Editors of the special issue (*i.e.*, Dr.'s A.Z.M. Salem, and Nicholas Odongo) were contacted and invited many potential authors directly. All abstracts received (46 abstracts) were assessed and were invited to submit full papers, while 24 full papers were only accepted after deep revision by two or more experts. All papers were reviewed by at least two primary reviewers chosen for their expertise in animal nutrition, especially in use of exogenous enzymes and probiotics in ruminants and non ruminant animals. Each one of the Guest Editors has handled some manuscripts and given the final decision with help from the reviewer's comments and evaluations. All papers were improved as a result of the reviewers' comments, indeed some were very extensively revised, and some were rejected.

Finally, the editors are very grateful for the time and commitment given by all the reviewers involved in the evaluation of the manuscripts submitted, and would like to acknowledge the excellent review efforts of: M.A. Cerrillo-Soto, Adibe L. Abdalla, Z.L. Tan, P. Micek, A. Cieslak, T. Szwaczkowski, J. Dach, S. Świątkiewicz, D. Józefiak, J. Skomiał, J. Mazurkiewicz, G. Paci, P. Huhtanen, D.F. Cardia, Z.M. Kowalski, S.S. Paul, N.P. Guerra, C. O'Shea, , S. Nowicki , A.S. Moura, J. Xin Liu, T. Seresinhe, D. Colombatto, H. Abubeker, J. Simbaya, H. Ben Salem, J. Rekhis, T. Norovsambuu, M. J. Ranilla, A. Abdalla, M. González-Ronquillo, M.D. Carro, M. Mellado, G.D. Mendoza, M. Cobos Peralta, M.S. Awawdeh, P.A. Hernández García, Z. Durmic, I.A. Domínguez-Vara, F. Klevenhusen, A.K. Patra, A. Kholif, A.M. Kholif, S. Gonzalez-Muñoz, Y. Rouzbehan. If any reviewer was missed, please accept our most sincere apologies.

Guest editors would like to acknowledge the technical and facilitations supports of IAEA/FAO, Vienna (Austria), to prepare this special issue.

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Animal Nutrition and Feed Technology (2013) 13: 337-353

Effect of Method of Adding a Fibrolytic Enzyme to Dairy Cow Diets on Feed Intake Digestibility, Milk Production, Ruminal Fermentation, and Blood Metabolites#

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ABSTRACT

Dean, D.B., Staples, C.R., Littell, R.C., Kim, S.C. and Adesogan, A.T. 2013. Effect of method of adding a fibrolytic enzyme to dairy cow diets on feed intake digestibility, milk production, ruminal fermentation, and blood metabolites. Animal Nutrition and Feed Technology, 13: 337-353.

This study investigated the effect of the method of applying a fibrolytic enzyme (Promote®; Cargill; Minnetonka, MN) on the performance of lactating dairy cows. A diet consisting of Tifton 85 bermudagrass silage, corn silage, and concentrate (35, 10 and 55% of dietary DM respectively) was fed *ad libitum* as a total mixed ration (TMR) twice daily. Cows were assigned randomly to the following five treatments: 1) control (no enzyme added), enzyme applied 2) at ensiling to bermudagrass (ES), 3) at mixing to the concentrate (EC), 4) at feeding to the TMR (ETMR), or 5) the bermudagrass silage (EF). In Experiment 1, thirty Holstein cows (129 days in milk, DIM) were used in a completely randomized, cross-over design consisting of two 28-d periods, with 14 d for adaptation and 14 d for sample collection. Voluntary DMI, total tract apparent digestibility of DM, NDF and CP, milk production and component yields were not affected by enzyme supplementation. Cows fed ETMR had lower blood β-hydroxybutyrate concentration, tended to have greater milk fat and protein concentrations and lower blood urea-N concentration than cows fed the control diet. In Experiment 2, five ruminally-cannulated cows were fed the same five diets for three consecutive 15-d periods to measure rumen fermentation (d 12) and *in situ* degradation (d 14 and 15). Compared to cows fed the control diet, ruminal pH tended to be lower in cows fed EC and ruminal NH_3-N concentration was lower in cows fed the ETMR diet. The molar proportion of acetic acid was lower in cows fed ETMR and EF diets vs. the control diet. Total volatile fatty acid concentration and acetate: propionate ratio were lower in cows fed ETMR vs the control diet. Enzyme application did not affect *in situ* degradability except that the ES diet tended to degrade at a faster rate than the control diet. Enzyme application to the TMR gave the most desirable responses (tended to increase milk fat and protein concentrations and improved the efficiency of ruminal energy utilization and decreased ruminal protein degradation). However, enzyme addition did not affect intake, digestibility or milk yield.

Key words: Bermudagrass, Dairy cows, Fibrolytic enzyme, Silage.

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

Several recent studies have evaluated the potential for improving diet utilization and dairy cow performance with exogenous fibrolytic enzymes. Enzyme products comprised mainly of xylanases and cellulases have increased digestibility in some studies (Rode *et al.*, 1999; Yang *et al.*, 2000) but not others (Lewis *et al.*, 1999). Consequently, enzyme addition has increased or tended to increase milk production in some studies (Rode *et al.*, 1999; Yang *et al.*, 2000, Kung *et al.*, 2002), but not in others (Vicini *et al.*, 2003). These discrepancies are likely due to differences in enzyme activity and composition, stage of lactation of cows, and method of enzyme addition.

Unlike abomasal or ruminal enzyme infusion (Hristov *et al.*, 2000), enzyme addition to the dietary forage (Lewis *et al.*, 1999) or concentrate (Rode *et al.,* 1999; Yang *et al.*, 2000) has improved the performance of cows, reflecting the importance of a close association between the feed and enzymes for fiber hydrolysis (Beauchemin *et al.*, 1999). However, few studies have determined whether enzymes are most effective when added to the dietary concentrate or forage. In theory, enzyme application to the dietary forage or TMR should be more effective than application to the concentrate because of greater opportunity for enzyme-induced fiber hydrolysis. Exogenous enzymes should also be more effective when applied to high-moisture feeds such as silages than to dry feeds because of the importance of water for enzymatic cell wall hydrolysis (Beauchemin *et al.,* 1999). However, one study has demonstrated that enzyme application to the concentrate was more effective than application to the TMR (Yang *et al.*, 2000). Whereas, Sutton *et al.* (2003) indicated that milk production was unaffected by the dietary fraction to which enzymes were applied. It is also thought that enzyme efficacy is positively correlated with the proportion of the diet to which enzymes are applied (Bowman *et al.*, 2002), but this is not always true (Yang *et al.*, 1999). These conflicting results emphasize the importance of further examination of how the method of enzyme application affects efficacy.

Enzyme application at or just prior to feeding is attractive because the products of cell wall hydrolysis can be directly fermented by ruminal bacteria, thereby releasing energy for the host animal, but care is needed to ensure an even distribution of the small quantity of enzyme that is added. Enzyme application at ensiling is an appealing alternative because uniform distribution throughout the forage can be ensured when enzymes are applied using properly calibrated sprayers on forage harvesters. There is also a sound theoretical basis for applying fibrolytic enzymes to forages at ensiling because they can hydrolyze plant cell walls into fermentable substrates that improve homolactic fermentation, while improving digestibility. Fibrolytic enzyme application at ensiling has improved the fermentation and nutritive value of corn silage (Colombatto *et al.*, 2001) and bermudagrass silage (Dean *et al.*, 2005). However, few, if any studies have compared how applying enzymes at ensiling versus at feeding affects the performance of dairy cows. Little is also known about whether fibrolytic enzyme

addition can improve milk production in dairy cows fed diets based on tropical grass silages. Despite their lower digestibility, such grasses are higher yielding and cheaper to produce or purchase than corn silage or alfalfa hay or silage. Therefore, they can be used to improve the profitability of dairy farming in tropical or subtropical regions, particularly if their nutritional values can be improved by enzyme addition. The objective of this study was to determine if four methods of enzyme application to diets based on BS improve feed intake, digestion, and milk production and composition by dairy cows. The methods included enzyme application to the forage at ensiling or at feeding, application to the concentrate at mixing, or application to the TMR at feeding. The enzyme used in this study was more effective than three other commercial enzymes at reducing DM losses and increasing cell wall hydrolysis to sugars, promoting a more homolactic fermentation, and increasing fiber digestibility of BS (Dean *et al.*, 2005). It had also increased digestibility and milk production when added to a dairy cow diet Rode *et al*. (1999).

MATERIALS AND METHODS

Cows and management

Two experiments were carried out at the Dairy Research Unit of the University of Florida from November 2004 to March 2005. In the first experiment, 5 primiparous and 25 multiparous lactating Holstein cows in mid-lactation (129±6 DIM) were grouped by parity and milk production. Cows within each group were allocated randomly to 5 dietary treatments for two consecutive 28-d periods. At the end of Period 1, cows were assigned randomly to a different treatment in period 2 in such a way that each treatment assignment followed another treatment from period 1 to 2 no more than twice. Each period consisted of 14 d for adaptation to a new diet and 14 d for sample collection. Cows were fed individually twice daily (0700 and 1430 h) using Calan gates (American Calan Inc., Northwood, NH). Feed refusals were collected daily at 0600 h and cows had free access to drinking water. Cows were retained in free stalls with sand as bedding and the barn was equipped with fans and misters to prevent heat stress. All experimental procedures complied with the guidelines of the University of Florida Institutional Animal Care and Use Committee.

Bermudagrass silage production and treatment

Tifton 85 bermudagrass was mowed after 35 d of regrowth using a CLAAS Disco 3000 TC forage mower (CLAAS of America, Omaha, NE), wilted for 2 h, and chopped (5-cm particle size) using a CLAAS Jaguar 9000 (CLAAS of America, Omaha, NE) forage harvester). Sixty-two tons of bermudagrass were packed into each of two, 3.6 m-wide Ag Bags (AG Bag International, Warrenton, OR) using a Versa Bagger (model ID 1012, Versa Corp., Astoria, OR) and stored for 35 d. A fibrolytic enzyme complex (Promote®, Cargill, Minnetonka, MN) was diluted in water (1:5 ratio v/v) and sprayed on 46 tons of bermudagrass forage at a rate of 1.3 g/kg DM as the

forage was being packed into an additional Ag-bag. The manufacturer stipulated that the main activity of promote is 1200 cellulase units/g, where one unit is the amount of enzyme that releases 1 μmol of glucose from cellulose in 1 min at 40°C. Cellulase activity also was determined at 39°C and pH 5.5 using the filter paper method (Wood and Bhat, 1988). The activity was found to be 38.4 filter paper units/g, where one unit of activity is the amount of enzyme that releases exactly 2 mg of glucose from 50 g of filter paper in 60 min.

Diets and treatments

The diets fed in both experiments contained Tifton 85 BS, corn silage, and concentrate mixed at 35, 10 and 55% of dietary DM, respectively (Table 1). The Promote enzyme was applied to different fractions of the diet to give the following treatments: 1) no enzyme added (Control), 2) enzyme applied to bermudagrass at ensiling (ES 1.3 g/kg DM), enzyme applied at feeding at the rate of 4 g/head/d 3) at mixing to the concentrate (EC), 4) at feeding to the TMR (ETMR) or 5) the forage (EF). Therefore, daily enzyme intake in the ES treatment (9 g/head/d) was greater than that used in other treatments, but the rate used at feeding was recommended by the manufacturer, while that used at ensiling was shown previously to be effective at improving silage quality (Dean *et al.*, 2005). Diets were mixed for 5 min prior to feeding in three 250-kg Calan data rangers (American Calan Inc., Northwood, NH). One data ranger was only used for the control diet, another for the ES diet, and a third

Table 1. Ingredient and chemical composition of the basal diet without enzyme supplementation (n=4)

Ingredient composition	% Dietary DM
Bermudagrass silage	35.0
Corn silage	10.0
Ground corn	27.0
Citrus pulp	5.1
Whole cottonseed	2.8
Soy Plus[1]	6.6
Soybean meal	8.6
Mineral mix[2]	4.4
Biophos (Calcium phosphate)[3]	0.4
Chemical composition:	
DM, %	61.8
CP, % of DM	16.2
ADF, % of DM	24.9
NDF, % of DM	39.8
TDN, % of DM	72.8
NEL, Mcal/kg of DM	1.40

[1]West Central Soy, Ralston, IA; [2]Mineral mix contained 26.4% CP, 10.2 Ca, 0.9% P, 3.1% Mg, 1.5% S, 5.1% K, 8.6% Na, 1698 mg/kg of Zn, 512 mg/kg of Cu, 339 mg/kg of Fe, 2231 mg/kg of Mn, 31 mg/kg of Co, 26 mg/kg of I, 7.9 mg/kg of Se, 147,756 IU of vitamin A/kg, 787 IU of vitamin E/kg (DM basis); [3]IMC Feed Ingredients, Lake Forest IL; contained 15.9% Ca, 21.2% P.

for diets to which enzyme application occurred just before feeding. The latter was washed with water between feeding treatments 3, 4 and 5 to avoid cross contamination.

The enzyme-treated concentrate was prepared weekly by dissolving the enzyme in water (1:5 ratio v/v) and spraying the solution on 140 kg of ground corn while the corn was being mixed in a Marion Mixer (Rapids Machinery Co., Marion, IA). The rest of the concentrate ingredients were mixed subsequently with the enzyme-treated corn in a 900 kg New Holland 355 mixer (New Holland North America, New Holland, PA). Untreated and treated concentrates were stored in 2.5 ton metal grain bins. For the EF and ETMR treatments, the enzyme was dissolved in water (1:10 ratio v/v) and sprayed on the forage and the TMR, respectively within a data ranger. The enzyme-treated feedstuffs were mixed subsequently for 5 min to ensure thorough distribution and then fed.

Sample collection and analysis

Cows were milked 3x daily at 0200, 1000, and 1800 and milk production (MP) was measured during the last 14 d of each period. Milk samples were collected twice daily (1000 and 1800) on two consecutive days during each week in the last 2 wk of each period and preserved with potassium dichromate. Milk samples were analysed by Southeast Milk lab (Belleview, FL) for concentration of fat, true protein and somatic cell counts (SCC) using a Bentley 2000 near infrared reflectance analyser (Bentley Instruments Inc., Chaska, MN). Feed efficiency was calculated based on milk production and DMI (kg of milk/kg DMI). Body weight and BCS ranging from 1 (thin) to 5 (obese) were measured (Wildman *et al.*, 1982) on three consecutive days after the 1000 h milking at the beginning and end of each period. Plasma samples (10 ml) were taken on the last day of each period using vacutainers (BD Vacutainer, Franklin Lakes, NJ) containing sodium heparin by coccygeal arterio-venipuncture and immediately placed on ice. Samples were centrifuged at 2500×g for 20 min at 4°C and the plasma was frozen at -20°C. Concentration of plasma glucose was determined using a Technicon Autoanalyser II (Bran-Luebbe, Elinsford, NY) with a modification of the method of Gochman and Schmitz (1972). Blood urea nitrogen (BUN) was determined using an autoanalyser method (Technicon Industrial systems Autoanalyser II; Industrial method # 339-01; Tarrytown, NY), which is an adaptation of the carbamido-diacetyl reaction described by Coulombe and Favreau (1963). Plasma concentration of β-hydroxybutyrate acid (BHBA) was determined using the procedure described by Williamson *et al.* (1962).

Chromic oxide was used for determination of apparent digestibility. Chromic oxide powder was weighed into gelatin capsules (Jorgensen Lab., Loveland, CO) and dosed twice daily via a balling gun (10 g/dose at 0700 and 1900 h) for 10 consecutive days in each experimental period. Faecal samples (approximately 100 g) were collected during the last 5 d of each period at the time of dosing. Faeces were dried to constant weight at 55°C in a convection oven, ground to pass through a 1-mm Wiley mill screen

(A.H. Thomas, Philadelphia, PA) and a composite sample was made from all ten faecal samples per cow per period. Chromium concentration in faeces was determined using a Perkin Elmer 5000 (Wellesley, MA) atomic absorption spectrometer according to the procedure described by Williams *et al.* (1962). The marker-ratio technique (Schneider and Flatt, 1975) was used to calculate apparent DM and nutrient digestibility. During each week of each collection period, four samples each of the concentrate, forages (corn silage and treated and untreated bermudagrass) and untreated TMR were collected, and stored (2°C), and subsequently composited and analysed. Therefore, the four samples of each ingredient that were analysed represented the feed offered each week in each of the two periods. These samples were sent to the Dairy One Forage Testing Laboratory (Ithaca, NY) for CP, NDF, ADF, and NEL analysis on all samples by wet chemistry, and NH_3-N, NFC, lactic acid, VFA and pH analysis on silage samples. The method of Van Soest *et al.* (1991) was used for ADF and NDF analysis and amylase and sodium sulfite were included in the NDF assay. Lactic acid and VFA were analysed with a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer Life and Analytical Sciences, Boston, MA) containing a 20 m, 2 m x 2 mm tightspec ID, 4% Carbowax column. Ammonia-N concentration was measured using the AOAC (1990) method and pH was measured with a Corning combination electrode pH meter (Corning Inc, NY).

In Experiment 2, five ruminally-cannulated cows were used to evaluate the effect of the dietary treatments on ruminal pH, VFA and ammonia-N concentration and *in situ* ruminal TMR degradation during three consecutive 15-d periods. Each period consisted of 12 d of adaptation to a new diet, 1 d of ruminal fluid collection and 2 days of *in situ* rumen DM degradability measurements, but the latter was only measured in periods 2 and 3. Ruminal fluid was collected (200 ml) by aspiration and filtered through two layers of cheesecloth at 0, 2, 4, 6, 8 and 10 h after feeding on d 13. The pH was measured immediately after ruminal fluid collection using a pH meter (Accumet, model HP-71, Fisher Scientific, Pittsburgh, PA), pH data were then transformed into H^+ concentration and statistically analysed (Murphy, 1982). After pH measurement, the ruminal fluid was acidified with 3 ml/sample of H_2SO_4 (50% v/v) and the mixture was centrifuged at 5000×g for 20 min, after which the supernatant was collected and frozen (-20°C) in 20 ml vials. Volatile fatty acids were measured using the method of Muck and Dickerson (1988) using a high performance liquid chromatograph (Hitachi®, FL 7485, Tokyo, Japan) coupled to a UV detector (Spectroflow 757, ABI Analytical Kratos Division, Ramsey, NJ) set at 210 nm. The column used was a Bio-Rad Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA 9454) model with 0.015M sulfuric acid mobile phase and a flow rate of 0.7 ml/min at 45°C. Ammonia N was determined with a Technicon Auto Analyzer (Technicon, Tarrytown, NY, USA) and an adaptation of the Noel and Hambleton (1976) procedure.

During the second and third periods, the DM degradation kinetics of the experimental diets were measured *in situ* by incubating TMR samples within nylon bags (50 μm pore size, Bar Diamond Inc., Pharma, ID) for 0, 2, 4, 6, 8, 24 and 48 h in the

rumen. Dried (65°C for 48 h) and ground (4 mm screen) TMR samples were weighed (5 g as is) into nylon bags in triplicate and incubated in cows fed the same diet during d 14 and 15 of each period. At each incubation time, bags were removed from the rumen, rinsed with cool water, and frozen. At the end of each period, all bags were thawed and washed through a rinse cycle without soap in a Kenmore Series 70 washing machine and dried for 48 h at 60°C. The *in situ* degradation parameters were described using the model of McDonald (1981):

$$P = a + b\,(1-e^{-c\,(t-L)})$$

Where, P=DM (%) degraded at time t, a=wash loss of DM (%) at time zero, b=potentially degradable DM fraction, a+b=total degradable fraction, kd=the rate at which b is degraded, t=ruminal incubation duration (h), and L=lag time before initiation of DM digestion. The constants a, b, kd, and L were estimated using the NLIN procedure of SAS (2002). Rumen DM degradability was calculated as kd/kp+kd; where kp=passage rate of 0.08 (NRC, 2001).

Statistical analysis

Both experiments involved partially balanced, incomplete block cross over designs and the data were analysed using Proc Mixed (SAS, 2002). The model used for analyzing the results from Experiment 1 and the *in situ* degradability results in Experiment 2 was the following:

$$Y_{ijk} = \mu + T_i + P_j + C_k + R_l + E_{ijkl;}$$

Where, μ=general mean; T_i=treatment effect (fixed effect); P_j=period effect (fixed); C_k=cow effect (random effect); R_l=residual effect or carry over effect of treatment in the previous period; E_{ijkl}=experimental error

Dummy variables were included in the linear model to correct for carry over effects (Littell *et al.*, 2006). For analysis of milk production, each cow's pretrial milk production was used as a covariate. Contrast statements were used to compare each of the enzyme treatments to the Control. The model used for analyzing ruminal pH, VFA, and ammonia data in Experiment 2 was the following:

$$Y_{ijk} = \mu + T_i + P_j + H_k + C_l + TH_m + E_{ijklm}$$

Where, μ: general mean; T_i: treatment effect (fixed effect); P_j: period effect (fixed); H_k: time effect (repeated measurement); C_l: cow effect (random effect); $TH_{m:}$ time x treatment interaction; E_{ijklm}: experimental error.

The AR (1) covariance structure was used and a slice statement was used to detect differences among treatments at each incubation time. Significance was declared at $P<0.05$ and tendencies at $P<0.15$.

RESULTS AND DISCUSSION

Chemical composition of the dietary ingredients

The quality of the bermudagrass forage and corn silage were typical of those produced under tropical conditions. Table 2 suggests that enzyme-treated BS had more CP, NFC, NEL, and organic acid concentrations and less fiber and NH_3-N than untreated BS. Although the addition of the enzyme at ensiling was not replicated, the data supports that of Dean *et al*. (2005) who concluded that the enzyme improved the fermentation of BS. The low organic acid concentrations, high pH, and ammonia-N concentration of the untreated silage may reflect reduced substrates for fermentation or utilization of fermentation products by aerobic organisms such as molds and lactate-utilizing yeasts. The latter explanation is more plausible because ensiled, untreated BS typically has higher organic acid concentrations and lower pH than reported in this study, and adding the enzyme at ensiling has reduced yeast and mold growth in BS (Dean *et al.*, 2005).

Voluntary intake and apparent digestibility

Enzyme addition did not affect the intake of DM, NDF or CP (Table 3). Rode *et al*. (1999) also found no intake response to Promote® supplementation to the concentrate in diets of cows in early lactation. Other proprietary enzyme products also have not improved the intake of dairy cows (Yang *et al.*, 2000; Kung *et al.*, 2002; Sutton *et al.*, 2003), though some have not (Lewis *et al.*, 1999). The method of enzyme addition has also had no effect on feed intake in previous studies (Yang *et al.*, 2000; Bowman *et al.*, 2002; Sutton *et al.*, 2003).

Apparent total tract digestibilities of DM, NDF and CP were unaffected by enzyme treatment (Table 3). This partly explains the lack of an intake response to enzyme application. In contrast, Rode *et al*. (1999) observed that digestibility of a corn silage-based TMR was increased by treatment with Promote®. Similarly Beauchemin *et al*. (1999) reported that treatment with four parts of Promote® and one part of a pectinase mixture increased total tract apparent OM digestibility of a barley silage-based TMR. Differences in digestibility responses between the latter studies and this one may be related to the presence of pectinase in the enzyme mixture of Beauchemin *et al*. (1999), the use of cows in early lactation by Rode *et al*. (1999), and greater NDF and lignin concentration in the basal forage in this study. Nevertheless, enzyme application has not improved digestibility in several other studies involving basal temperate silages with relatively low NDF and lignin concentrations (Lewis *et al.*, 1999; Sutton *et al.*, 2003).

Table 2. Chemical composition of the enzyme-treated[†] and untreated forages and concentrates (% DM) (n=4 replicates per mean).

Item	DM, %	CP	NDF	ADF	TDN	NFC[‡]	NDF dig[§]	pH	NEL[ᵀ]	NH_3-N[ᵗ]	Lactic acid	Acetic acid	Propionic acid	Butyric acid
Untreated concentrate	88.3	21.9	15.8	8.55	84.5	-	-	-	1.98	-	-	-		-
Treated concentrate	88.4	21.5	15.6	8.15	84.0	-	-	-	1.97	-	-	-		-
Pooled SE	0.3	0.5	2.1	0.87	1.27			-	0.06					
Pre-ensiled BS	23.4	12.7	76.1	45.3	43.0	7.85	-	-	0.58	-	-	-	-	-
SE	3.0	0.9	1.1	2.2	1.5	0.4	-	-	0.05					
Corn silage	28.1	8.8	45.2	27.0	79.0	38.1	54.0	-	1.52	12.0	3.08	6.68	0.89	0.07
SE	4.2	1.2	1.5	3.1	2.1	0.6	4.0	-	0.08	15.4	0.60	0.35	0.034	0.03
Untreated BS[¶]	29.9	9.3	81.8	49.9	52.7	5.3	47.8	8.4	0.45	38.0	0.10	0.05	0.00	0.00
Enzyme-treated BS	29.6	11.4	76.2	45.2	55.0	8.6	49.3	4.6	0.77	13.7	1.77	3.08	0.32	0.18
Pooled SE	0.6	0.3	0.8	0.6	0.18	0.5	2.0	0.3	0.04	1.3	0.30	0.15	0.06	0.01

[†]Promote®, Cargill, Minnetonka, MN; [¶]Bermudagrass silage; [‡]Non-fiber carbohydrates;

[§]NDF digestibility; [ᵀ]Net energy of lactation; [ᵗ]As percentage of total N.

Table 3. Effect of method of enzyme[†] addition on voluntary intake and apparent digestibility

Treatment[‡]	Intake				Digestibility		
	DM, kg/d	DM, % BW	NDF, kg/d	CP, kg/d	DM, %	NDF, %	CP, %
Control (C)	20.9	3.35	9.7	3.4	66.4	50.7	65.6
EC	21.6	3.46	9.9	3.5	64.2	51.0	65.7
ETMR	22.4	3.65	10.0	3.5	66.3	50.4	66.9
EF	19.9	3.18	9.0	3.1	64.3	51.6	65.7
ES	21.8	3.41	9.5	3.3	68.3	48.7	67.4
SE	1.0	0.18	0.6	0.2	1.5	2.3	1.5
	P values						
Treatment effect	0.30	0.40	0.37	0.37	0.14	0.92	0.87
Contrasts							
C vs. EC	0.56	0.63	0.62	0.62	0.93	0.92	0.97
C vs. ETMR	0.22	0.21	0.22	0.22	0.78	0.93	0.55
C vs. EF	0.40	0.48	0.44	0.44	0.80	0.76	0.96
C vs. ES	0.45	0.80	0.72	0.72	0.50	0.55	0.40

[†]Promote®, Cargill, Minnetonka, MN

[‡]C: Control, EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, ES: enzyme-treated silage

Milk production and composition

Cows fed the Control diet tended ($P<0.15$) to produce more milk than those fed EC (Table 4). This result agrees with that of Beauchemin *et al.* (1999) on a barley silage-based TMR, and may be attributable to the lower ruminal pH and greater BW gain in cows fed EC in this study. Enzyme addition to concentrates rather than to other dietary components also increased area under pH 5.5 in the study of Bowman *et al.* (2003) but milk production was not reported. Cows fed EF also produced less milk than those fed the Control diet for unknown reasons. In contrast, Yang *et al.* (2000) reported greater milk production when fibrolytic enzymes were applied to the concentrate portion of a corn silage and alfalfa hay-based TMR. That enzyme application did not improve milk production also contradicts certain studies in the literature where enzyme addition increased or tended to increase milk production (Lewis *et al.*, 1999; Schingoethe *et al.*, 1999).

It is important to note that many of the published, beneficial milk production responses to fibrolytic enzyme addition to dairy cows diets have been tendencies or non-significant. This may be because some enzymes are developed and tested at pH and temperature optima that differ from those in the rumen (Beauchemin *et al.*, 2003; Vicini *et al.*, 2003). Therefore, future efforts should be directed at developing enzymes that exhibit optimal activity at normal rumen pH and temperature. Schingoethe *et al.* (1999) noted that dietary enzyme addition increased ($P<0.05$) milk production in cows in early lactation (<100 DIM) by 9 to 15%, but did not affect that of cows in midlactation (>100 DIM). Beauchemin *et al.* (1999) also reported that when cows were in positive

energy balance, enzyme-induced increases in digestible energy intake did not affect milk production. Therefore, the advanced stage of lactation (129 DIM) of cows in this study may have affected the milk response to enzyme addition.

Application of fibrolytic enzymes to the concentrate instead of the TMR increased total tract DM digestion and milk yield in one study (Yang *et al.*, 2000), decreased digestibility and did not affect milk yield in another one (Sutton *et al.*, 2003), and had no effect on these measures in this study.

Table 4. Effect of method of enzyme[†] addition on milk production and composition

Treatment[‡]	Milk, kg/d	4% FCM, kg/d	Milk fat, %	Milk fat, kg/d	Milk protein %	Milk protein, kg/d	kg milk/ kg DMI	SCC[3] x 10^3 cells/ml
Control (C)	33.1	31.8	3.67	1.23	2.91	0.96	1.64	339
EC	30.9	29.9	3.78	1.16	3.07	0.95	1.46	488
ETMR	32.3	32.4	3.99	1.29	3.07	1.00	1.42	581
EF	31.2	30.0	3.77	1.16	3.03	0.93	1.64	817
ES	32.3	30.6	3.72	1.19	2.90	0.92	1.59	458
SE	1.0	1.0	0.12	0.05	0.07	0.03	0.04	250
			P values					
Treatment effect	0.43	0.27	0.42	0.20	0.26	0.24	0.26	0.53
Contrasts								
C vs. EC	0.10	0.17	0.53	0.28	0.08	0.82	0.15	0.26
C vs. ETMR	0.52	0.68	0.07	0.33	0.08	0.24	0.08	0.53
C vs. EF	0.13	0.18	0.59	0.27	0.19	0.50	0.96	0.10
C vs. ES	0.51	0.40	0.79	0.50	0.92	0.35	0.70	0.58

[†]Promote®, Cargill, Minnetonka, MN

[‡]C: Control, EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, ES: enzyme-treated silage; [3]SCC: somatic cell counts.

Cows fed ETMR tended ($P<0.09$) to have greater milk fat and protein concentration than cows fed the control diet. Cows fed EC also tended ($P=0.076$) to have greater concentration of milk protein than those fed the control diet (Table 4). Several studies have not shown a milk composition response to dietary enzyme addition (Lewis *et al.*, 1999; Kung *et al.*, 2002) but some have (Schingoethe *et al.*, 1999; Sutton *et al.*, 2003) and the reasons for the differences are not clear. For unknown reasons, but as reported by Schingoethe *et al.* (1999), SCC tended to be greater in cows fed EF. The efficiency of feed utilization for milk production tended ($P=0.082$) to be lower in cows fed ETMR vs. the control diet.

Plasma glucose, urea-N and β-hydroxybutyrate

Cows fed EF and ES had lower ($P<0.05$) BUN concentrations than those fed the control diet and cows fed ETMR had a similar tendency ($P=0.123$; Table 5). This suggests that these treatments increased the efficiency of N utilization by ruminal microbes since N intake was similar among treatments.

In support of the results of Hristov *et al*. (2000), the ETMR treatment reduced ($P<0.01$) BHBA concentration, which is an indicator of increased fat mobilization or increased energy balance due to improved oxidation of ketone bodies in early stages of lactation. However, the cows in this study were in midlactation and they had positive energy balances that were unaffected by treatment (mean$=10.02\pm0.24$ Mcal/day).

Table 5. Effect of method of enzyme[†] addition on BW, BW gain, BCS, and blood metabolite concentration

Treatment[‡]	Variable					
	BW, kg	BW gain, kg/d	BCS	BUN, mg/dl	Glucose, mg/dl	βHBA[3], mM/l
Control (C)	633	0.20	2.82	16.9	64.5	0.94
EC	635	0.64	2.61	16.0	62.9	0.84
ETMR	624	0.42	2.77	15.6	64.6	0.68
EF	618	0.21	2.64	15.2	64.5	0.86
ES	623	0.31	2.83	15.2	64.5	0.83
SE	19	0.20	0.12	0.6	1.1	0.07
	P values					
Treatment effect	0.96	0.46	0.48	0.23	0.77	< 0.01
Contrasts						
C vs. EC	0.94	0.14	0.18	0.28	0.29	0.46
C vs. ETMR	0.72	0.45	0.74	0.12	0.97	< 0.01
C vs. EF	0.57	0.98	0.25	0.05	0.99	0.27
C vs. ES	0.72	0.70	0.96	0.05	0.98	0.14

[†]Promote®, Cargill, Minnetonka, MN

[‡]C: Control, EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, ES: enzyme-treated silage; [3]βHBA: beta hydroxybutyrate.

Ruminal pH and concentration of VFA and NH_3-N

Mean ruminal fluid pH tended to be only lower ($P=0.109$) in cows fed EC vs the Control diet, presumably due to greater enzymatic hydrolysis of the concentrate than the forage into readily fermentable substrates that depress pH when fermented (Table 6). Ruminal pH fell below 6 within 6 h of feeding in cows fed EC and remained at 5.5 between 8 and 10 h after feeding (Fig. 1). A pH of 5.2-5.6 indicates sub-clinical ruminal acidosis in dairy cows (Stone, 2004), therefore, cows fed EC may have experienced bouts of sub-clinical ruminal acidosis that may have contributed to their lower milk production. Enzyme addition to concentrates rather than other dietary components also increased area under pH 5.5 in the study of Bowman *et al*. (2003).

Mean ruminal NH_3-N concentration was lower in cows fed ETMR ($P<0.01$) than in cows fed the control diet. This supports the tendency for lower BUN concentration in cows fed ETMR, and suggests that there was enhanced uptake of NH_3-N by ruminal microbes in cows fed ETMR. This may be attributable to greater fermentable metabolizable energy availability as suggested by the tendency ($P=0.07$) for greater molar proportion of propionic acid from this diet.

Mean total VFA concentration was lower ($P<0.05$) in cows fed ETMR, EF and ES rather than the control diet. The VFA responses in this study did not reflect the total-tract digestibility responses because VFA concentrations reflect ruminal fermentation alone, and ignore the contribution of intestinal digestion to total tract digestion.

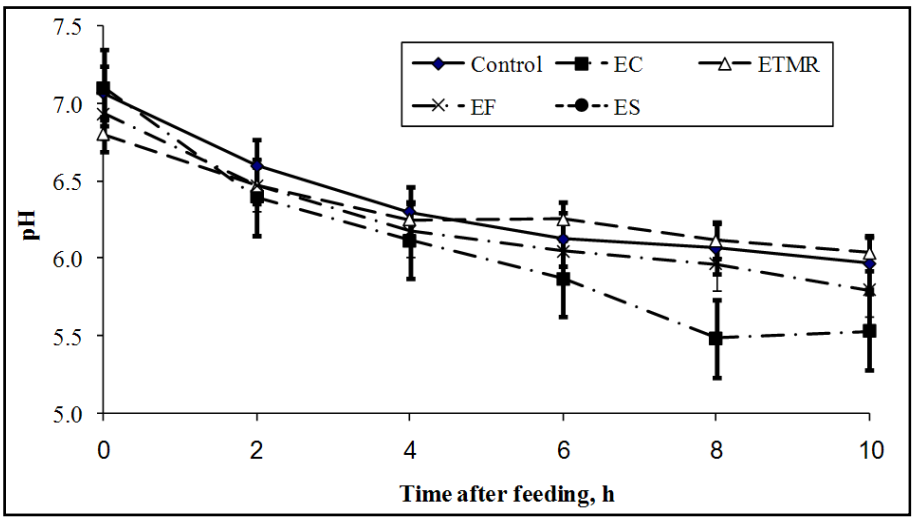

Fig. 1. Effect of method of enzyme addition to diet on ruminal fluid pH after feeding (n=3). EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, ES: enzyme-treated silage. X: Treatment means differed ($P<0.01$) at the specified time.

Ruminal molar proportion of acetic acid was lower in cows fed ETMR ($P<0.05$) and EF ($P<0.01$) than in those fed the control diet. Acetate: propionate ratio of ruminal fluid was lower ($P<0.05$) in cows fed ETMR vs the control diet. This lower acetate: propionate ratio typifies the effects of supplementation with ionophores on ruminal energetic efficiency, and implies that less energy was wasted as CO_2 and methane, and more net energy was available (NRC, 2001) from the ETMR diet. This may have contributed to the lower plasma BHBA concentration and tendency for higher milk constituent concentrations in cows fed ETMR. Increased supply of energy or propionate, which is a glucogenic oxaloacetate precursor, can increase milk protein concentration (Sutton, 1989) and enhance ketone body oxidation thereby reducing plasma BHBA concentration (Oba and Allen, 2003).

Only the ETMR diet reduced ruminal acetate: propionate ratio and NH_3-N concentration, and tended to increase milk fat and protein concentration. The exact reason why only the ETMR treatment had these effects is unknown, though it may reflect enzyme application to the greatest portion of the diet in this treatment (Bowman, 2002), thus allowing greater enzyme-diet interaction.

Table 6. Effect of method of enzyme† addition on ruminal pH and concentrations of VFA (mmol/100 mmol) and NH_3-N (mg/dL).

Treatment‡	pH	NH_3-N, mg/dL	Acetate C2, mol %	Propionate C3, mol %	Butyrate mol %	C2: C3 ratio	Iso-butyrate mol %	Valerate mol %	Iso- Valerate mol %	Total VFA mmol/L
Control (C)	6.32	15.1	58.9	20.8	11.8	2.8	2.8	3.5	4.0	144
EC	6.11	13.6	57.7	20.5	12.7	2.8	2.4	2.8	3.8	125
ETMR	6.27	10.1	56	21.7	12.3	2.6	2.6	3.7	3.8	105
EF	6.26	17.0	55.3	20.8	12.6	2.7	2.6	4.7	4.1	115
ES	6.23	14.9	59.2	20.2	11.7	2.9	2.4	3.1	3.3	106
SE	0.09	1.12	0.91	0.33	0.67	0.05	0.22	0.75	0.45	12.5
P values										
Treatment effect	0.53	0.01	0.02	0.07	0.78	< 0.01	0.70	0.42	0.72	0.07
Time effect	0.01	< 0.01	0.04	0.10	0.46	< 0.01	0.05	0.55	0.42	0.51
Treatment x time effect	0.45	0.01	0.78	0.92	0.91	0.99	0.45	0.53	1.00	0.92
Contrasts										
C vs. EC	0.11	0.40	0.38	0.58	0.34	0.75	0.23	0.52	0.71	0.25
C vs. ETMR	0.73	<0.01	0.03	0.07	0.59	0.02	0.52	0.83	0.70	0.02
C vs. EF	0.63	0.29	0.01	0.99	0.38	0.12	0.40	0.23	0.92	0.08
C vs. ES	0.52	0.97	0.78	0.20	0.95	0.05	0.20	0.73	0.26	0.02

†Promote®, Cargill, Minnetonka, MN;

‡C: Control, EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR,

EF: enzyme applied to forage at feeding, ES: enzyme-treated silage.

In situ DM disappearance

The rate of degradation and rumen degradability of ES tended to be greater (P = 0.11) than that of the Control diet (Table 7). Unlike those for ES, the *in situ* degradability results for ETMR, EF and EC diets agree with the *in vivo* total tract digestibility results and with other studies showing no effects of enzyme application on forage degradability (Adesogan *et al.*, 2005). The *in situ* results for ES agree with Dean *et al*. (2005) who showed that application of the same enzyme at the same rate to bermudagrass at ensiling, increased *in vitro* DM and NDF digestibility.

Table 7. Effect of method of enzyme[†] addition on the kinetics of *in situ* DM disappearance of a TMR in lactating cows

Treatments[‡]	Parameters[§]						
	a, %	b, %	a+b, %	P, %	c,/h	L, h	RD[¶],%
Control (C)	40.3	38.9	82.4	74.7	0.061	2.6	42.9
EC	36.2	38.5	78.2	72.5	0.058	3.6	42.0
ETMR	39.3	38.4	80.5	73.2	0.063	2.3	44.0
EF	36.2	36.6	75.0	59.0	0.079	4.2	48.6
ES	36.4	40.9	77.8	68.3	0.112	5.6	57.1
SE	3.5	5.7	5.4	11.6	0.025	3.7	5.2
P values							
Treatment effect	0.54	0.94	0.72	0.69	0.34	0.59	0.34
Contrasts							
C vs. EC	0.31	0.99	0.47	0.85	0.93	0.65	0.91
C vs. ETMR	0.72	0.95	0.75	0.90	0.92	0.91	0.89
C vs. EF	0.32	0.58	0.29	0.24	0.51	0.49	0.47
C vs. ES	0.16	0.83	0.44	0.61	0.11	0.22	0.11

[†]Promote®, Cargill, Minnetonka, MN

[‡]C: Control, EC: enzyme applied to concentrate, ETMR: enzyme applied to the TMR, EF: enzyme applied to forage at feeding, ES: enzyme-treated silage.

[§]a: wash loss fraction, b: insoluble but potentially degradable fraction, a+b= total degradability, P:DM degraded at time t, c: rate of degradation of b fraction, L: lag phase.

[¶]Rumen DM degradability adjusted for rate of passage (NRC, 2001).

CONCLUSIONS

None of the methods of fibrolytic enzyme addition improved milk production, milk constituent yields, *in situ* degradability, *in vivo* digestibility, or voluntary intake. However, enzyme addition to the TMR treatment reduced the ruminal acetate to propionate ratio, decreased concentrations of plasma BHBA and ruminal ammonia-N, tended to reduce BUN concentration and to improve milk fat and protein concentrations. Enzyme addition to the concentrate tended to reduce milk yield and ruminal pH, and increase BW gain and milk protein concentration. Enzyme addition to the forage at feeding tended to increase SCC and decrease concentrations of ruminal acetate, total

VFA, and BUN. Enzyme addition at ensiling tended to increase the ruminal degradation rate, and decreased total VFA and BUN concentrations. Therefore, the method of enzyme application with the most desirable effects was addition to the TMR, but none of the methods of enzyme application improved milk production by the cows.

REFERENCES

Adesogan, A.T., Krueger, N.A. and Kim, S.C. 2005. A novel, automated system for measuring fermentation gas production kinetics of feeds and its application to feed characterization. *Animal Feed Science and Technology,* 123-124: 211-223

AOAC 1990. Urea and ammoniacal nitrogen in animal feed. In: *Official Methods of Analysis of the Association of Official Analytical Chemists International*, 15th ed., Method 941.04. Arlington, VA, USA. p. 76

Beauchemin, K.A., Yang, W.Z. and Rode, L.M. 1999. Effect of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. *Journal of Dairy Science,* 83: 378-390.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science,* 81 (E. Suppl. 2): E37-E47.

Bowman, G.R., Beauchemin, K.A. and Shelford, J.A. 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by dairy cows. *Journal of Dairy Science,* 85: 3420-3429.

Bowman, G.R., Beauchemin, K.A. and Shelford, J.A. 2003. Fibrolytic enzymes and parity effects on feeding behavior, salivation, and ruminal pH of lactating dairy cows. *Journal of Dairy Science,* 86: 565-575.

Colombatto, D., Mould, F.L., Bhat, M.K., Phipps, R.H. and Owen, E. 2001. Effects of ensiling temperature and enzyme additives on the fermentation and *in vitro* rumen degradation of maize silage. *Journal of Dairy Science,* 84 (Suppl.1): 424-425.

Coulombe, J.J. and Favreau, L. 1963. A new simple semi micro method for colorimetric determination of urea. *Clinical Chemistry,* 9: 102-108.

Dean, D.B., Adesogan, A.T., Krueger, N. and Littell, R.C. 2005. Effect of fibrolytic enzymes on the fermentation characteristics, aerobic stability, and digestibility of bermudagrass silage. *Journal of Dairy Science,* 88: 994-1003.

Gochman, N. and Schmidz, J.M. 1972. Application of a new peroxide indicator reaction to the specific, automated determination of glucose with glucose oxidase. *Clinical Chemistry,* 18: 943-952

Hristov, A.N., McAllister, T.A. and Cheng, K.J. 2000. Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: Effects on nutrient digestion in cattle fed a barley grain diet. *Journal of Animal Science,* 78: 477-487.

Kung, L. Jr., Cohen, M.A., Rode, L.M. and Treacher, R.J. 2002. The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ration to lactating dairy cows. *Journal of Dairy Science,* 85: 2396-2402.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Prichard, G.T., Swanson, B.I. and Treacher, R.J. 1999. Effect of direct fed fibrolytic enzymes on the lactational performance of the dairy cow. *Journal of Dairy Science,* 82: 611-617.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D. and Schabenberber, O. 2006. *SAS for Mixed Models*. Second edition. SAS Institute, Incorporated., Cary, NC., USA.

McDonald, L. 1981. A revised model for the estimation of protein degradability in the rumen. *Journal of Agricultural Science,* 96: 251-252

Muck, R.E. and Dickerson, J.T. 1988. Storage temperature effects on proteolysis in alfalfa silage. Transactions of the ASAE, 31: 1005-1009.

Murphy, M.R. 1982. Analyzing and presenting pH data. *Journal of Dairy Science,* 65: 161-163.

Noel, R.J. and Hambleton, L.G. 1976. Collaborative study of a semi automated method for determination of crude protein in animal feeds. *Journal of the Association of Official Analytical Chemists,* 59: 134-140.

Oba, M. and Allen, M.S. 2003. Dose-response effects of intrauminal infusion of propionate on feeding behavior of lactating cows in early or midlactation. *Journal of Dairy Science,* 86: 2922-2931.

Rode, L.M., Yang, W.Z. and Beauchemin, K.A. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. *Journal of Dairy Science,* 82: 2121-2126.

SAS. 2002. *User's Guide: Statistics*. SAS Institute, Incorporated., Cary, NC. USA.

Schingoethe, D.J., Stegeman, G.A. and Treacher, R.J. 1999. Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. *Journal of Dairy Science,* 82: 996-1003.

Schneider, B.H. and Flatt, W.P. 1975. The indicator method. In: *The Evaluation of Feeds through Digestibility Experiments*, University of Georgia Press, Athens. p. 168.

Stone, W.C. 2004. Nutritional approaches to minimize subacute ruminal acidosis and laminitis in dairy cattle. *Journal of Dairy Science,* 87: E13-E26

Sutton, J.D. 1989. Altering milk composition by feeding. *Journal of Dairy Science,* 72: 2801-2814

Sutton, J.D., Phipps, R.H., Beever, D.E., Humphries, D.J., Hartnell, G.F., Vicini, J.L. and Hard, D.L. 2003. Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in holstein-Friesian cows. *Journal of Dairy Science,* 86: 546-556.

Vicini, J.L., Bateman, H.G., Bhat, M.K., Clark, J.H., Erdman, R.A., Phipps, R.H., Van Amburgh, M.E., Hartnell, G.F., Hintz, R.L. and Hard, D.L. 2003. Effect of feeding supplemental fibrolytic enzymes or soluble sugars with malic acid on milk production. *Journal of Dairy Science,* 86: 576-585.

Wildman, E.E., Jones, G.M., Wagner, P.E., Boman, R.L., Troutt, H.F.Jr. and Lesch, T.N. 1982. A dairy cow body condition scoring system and its relationship to selected production characteristics. *Journal of Dairy Science,* 65: 495-501.

Williams, C.H., David, D.J. and Iismaa, O. 1962. The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. *Journal of Agricultural Science,* 59: 381-385.

Williamson, D.H., Mellanby, J. and Krebs, H.A. 1962. Enzymatic determination of D (-) B-hydroxybutyric acid and acetoacetic acid in blood. Biochem. J. 228: 727-733.

Wood, T.M. and Bhat, M.K. 1988. Methods for measuring cellulase activities. In: *Methods in Enzymology*. (Eds. W.A. Wood and S.T. Kellog), Academic Press Inc., London, UK. Vol. 160: pp. 87-112.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3568-3597.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 1999. Effects of enzyme feed additives on extent of digestion and milk production of lactating dairy cows. *Journal of Dairy Science,* 82: 391-403.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science,* 83: 2512-2520.

Animal Nutrition and Feed Technology (2013) 13: 355-362

Evaluation of High Doses of Exogenous Fibrolytic Enzymes in Lambs Fed an Oat Straw Based Ration#

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ABSTRACT

Lara Bueno, A., Mendoza Martínez, G.D., Hernández García, P.A., Martínez García, J.A. and Plata Pérez, F.X. 2013. Evaluation of high doses of exogenous fibrolytic enzymes in lambs fed an oat straw based ration. Animal Nutrition and Feed Technology, 13: 355-362.

High doses of exogenous fibrolytic enzymes (EFE) were evaluated for their effects on lamb performance and *in situ* digestibility using a ration with 60% oat straw. Twenty-four lambs were used (25.6±3.05 kg initial weight) and distributed in a completely randomized design with three treatments that consisted of doses of 0, 5 or 10 g of EFE kg^{-1} DM oat straw. Intake decreased linearly ($P<0.04$) with increasing doses of EFE, without changing the weight gain, feed conversion, digestibility and ruminal fermentation variables. The results indicate that using high doses of EFE in oat straw based diets did not improve the growth performance and nutrient digestibility in finishing lambs.

Key words: Digestibility, Fibrolytic enzymes, Growth performance, Sheep.

INTRODUCTION

Intensive sheep feeding systems to finishing are dependent on the energy content of the diet, so these systems base their efficiency ratios on rations with high grain contents (Mendoza *et al.*, 2007). Recently, cereal costs have increased, due mainly to prolonged drought conditions and reduced global production as well as their diversion to the generation of biofuels (Chuck-Hernández *et al.*, 2011). This has led to feeding strategies that seek to replace the grain, without changing the productive response in sheep, by increasing fodder, which is one of the main sources of feed for ruminants; however, high proportions in the diet may limit productivity because the cell walls are not fully degraded by endogenous enzymes in the rumen due to acidic conditions created by feed concentrates (Zinn and Ware, 2002). To obtain the most nutrients from forages,

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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exogenous fibrolytic enzymes have been used, since there is evidence that their addition to diets for ruminants have positive effects on carbohydrate degradation of cell walls (Krueger *et al.*, 2008; Tang *et al.*, 2008), cooperating in the feed hydrolysis; they also interact synergistically with rumen micro-organisms to enhance the digestion of feed ingredients (Beauchemin *et al.*, 2004). However, when using high lignocellulolytic ingredients (agricultural wastes), the results have been variable and inconsistent in terms of fibre digestibility (Wang *et al.*, 2004; Colombatto *et al.*, 2007; Gallardo *et al.*, 2010) because the potential of exogenous enzymes depends mainly on the dose and type of substrate. So it is possible that lower quality forages require higher doses of exogenous fibrolytic enzymes in order to show benefits such as those observed with higher quality forage (Eun *et al.*, 2008). Therefore, the objective of this research was to evaluate the effect of adding different doses (0, 5 or 10 g) of exogenous fibrolytic enzyme kg^{-1} of oat straw on diet digestibility and growth performance of lambs fed a 60% ration of oat straw and 40% concentrate.

MATERIALS AND METHODS

This study was conducted at the Experimental Station "The Thirteen" of Chapingo, Huitzilac located in Morelos, Mexico. The experiment was conducted under the guidelines provided by the Academic Committee of the Department of Animal Science, according to the regulations established by the Animal Protection Law of Mexico, using 24 cross-breed sheep (Pelibuey with Canelo x Katadin) of 25.6±3.05 kg initial live weight. Lambs were housed in pairs in 12 experimental pens (experimental unity), for random distribution to three treatments with four replications. Treatments consisted of three doses of enzyme (0, 5 and 10 g enzyme per kg of forage). The sheep received a diet with a 60: 40 ratio of forage to concentrate, which was formulated considering the recommendations of the NRC (1985) based on the following percentages (%): oat straw (60), corn grain (8), grain sorghum (12), soybean meal (6), molasses (4), polished rice (4), mineral and vitamin premix (1), sodium bicarbonate (0.5), and urea (0.5). The diet composition was 84.9% dry matter, 13.6% crude protein, 40.5% neutral detergent fibre and 23.8% acid detergent fibre.

The rations were offered twice daily (09:00 and 17:00). The exogenous fibrolytic enzyme mix (EFE) employed was commercial (Fibrozyme TM, Alltech Inc., Nicholasville, KY, USA), obtained from fungal fermentation and possessing a xylanolytic activity of 100 IU of xylose g^{-1} product, and Meraz-Romero *et al.* (2012) reported an activity of 292 IU of xylanase g^{-1} and 36 IU g^{-1} of cellulases from this enzyme product. Treatments consisted of three doses of enzyme (0, 5 and 10 g enzyme kg^{-1} forage), sprayed directly on the oat straw. The experimental trial lasted 56 days, starting 8 days prior to allow adaptation to the ration. The animals were weighed at the beginning and at the end of the fasting experiment, to determine the average daily gain (ADG). Dry matter intake (CMS) was obtained by the difference between the feed offered and rejected. These data enabled estimation of the feed conversion (FC). Day 28

of the experiment, each animal's faeces were collected directly for the determination of total digestibility of dry matter, using the acid insoluble ash technique (van Keulen and Young, 1977), and subsequent determination of the NDF digestibility (Van Soest *et al.*, 1991). On the last day of the experiment, 50 ml of ruminal fluid was obtained from each sheep's preprandial rumen using a stomach tube, to determine effect of treatment on the rumen fermentation parameters. After determining the pH, the remainder of the rumen fluid was preserved by the addition 2 ml of 25% metaphosphoric acid (W/V) for later VFA concentration using the methodology proposed by Erwin *et al.* (1961) and following the technique of McCullough (1967), to determine the content of NH_3-N.

Moreover, the same treatments were used to determine the *in situ* digestibility of the cell walls of oat straw. For this purpose 5 g forage, with a particle size of 2 mm, which was previously treated with 0, 5 and 10 g kg^{-1} EFE forage treatments, were incubated in triplicate for 24, 48, 72 and 96 h in a fistulated cow, which used as incubator. After the incubation period, the bags were removed from the rumen and washed until the effluent was completely clear, dried at 60°C for 24 h, and then the cell wall contents were determined by the method of Van Soest *et al.* (1991).

The data obtained from the performance test and *in situ* digestibility were analysed using a completely randomized design employing the GLM procedure of SAS (Herrera and Garcia, 2010) and compared using Tukey's test, plus a linear effect and quadratic for treatment response (Steel *et al.*, 1997).

RESULTS AND DISCUSSION

No changes were observed ($P>0.05$) in the average daily gain and feed conversion; the only linear effect was found ($P=0.04$) in feed intake. There were no differences ($P>0.05$) in the *in vivo* digestibility of DM and NDF (Table 1). High doses of fibrolytic enzymes produced no changes ($P>0.05$) in pH and NH_3-N-; however, the concentration of volatile fatty acids (VFA) was modified, showing a quadratic effect ($P=0.04$). Propionic showed an effect ($P=0.07$), just as to propionic acid and acetic acid ratio ($P=0.06$) (Table 1). A quadratic response was observed for the *in situ* digestibility of the cell walls ($P<0.01$) after 48 hours of incubation; however, there were no changes in response to high doses of exogenous fibrolytic enzymes at the other incubation times (Table 2).

The results obtained in this study are consistent with several authors who assessed low-dose (1.5 g kg^{-1} EFE forage) exogenous enzymes with moderate proportions of forage by digestibility tests and changes in the behaviour of sheep (Almaraz *et al.*, 2010) and cattle (Kruegen *et al.*, 2008, Alvarez *et al.*, 2009; Eun *et al.*, 2009). Similarly, Varlyakov *et al.* (2010) reported that the addition of an enzyme complex containing commercial cellulases, β-glucanases, amylases and proteases, at a dose of 1 g kg^{-1} of substrate, with a low proportion of fodder (40%), did not affect dry matter intake and average daily gain; however, it improved feed conversion in sheep. This

Table 1. Effects of exogenous fibrolytic enzyme dose (EFE) on production variables and rumen of sheep.

Items	EFE, g kg^{-1} of feed			SEM	*P*	
	0	5	10		Lin	Qua
Productive performance						
Initial weight, kg	24.40	26.31	25.97	1.17	0.35	0.44
Final weight, kg	31.91	33.17	34.30	1.20	0.88	0.33
DMI, kg d^{-1}	1.10^{b}	1.19^{ab}	1.23^{a}	0.03	0.04	0.87
ADG, kg^{-1}	0.134	0.122	0.148	20	0.88	0.33
FC	10.17	10.61	9.39	1.55	0.76	0.63
Total digestibility						
TDDM, %	74.41	71.78	73.37	1.95	0.71	0.39
TDNDF, %	54.18	45.86	54.72	4.07	0.35	0.44
Ruminal parameters						
pH	6.24	6.44	6.37	0.11	0.43	0.37
N-NH$_3$, mg dL-1	8.80	7.31	7.64	1.98	0.68	0.71
Acetic (A), %	70.50^{ab}	69.37^{b}	71.08^{a}	0.51	0.43	0.04
Propionic (P), %	17.31	18.06	16.50	0.50	0.27	0.07
Butyric, %	12.18	12.55	12.41	0.34	0.64	0.55
A: P relationship	4.09	3.87	4.31	0.13	0.27	0.06

DMI: dry matter intake; ADG average dairy gain; FC: Food conversion ratio TDDM: Total digestibility dry matter; TDNDF: Total digestibility neutral fibre detergent.

SEM: Standard error of the mean; Lin: linear effect; Qua: quadratic effect

^{ab}Means with different letters are significantly different ($P<0.05$).

Table 2. *In situ* digestibility of the fibre with the addition of high doses of exogenous fibrolytic enzymes (EFE) in sheep diets.

Incubation, h	EFE, g kg^{-1} of feed			SEM	*P*	
	0	5	10		Lin	Qua
24	33.42	35.44	35.03	1.46	0.55	0.44
48	37.66^{b}	41.39^{a}	41.20^{a}	0.59	0.06	0.01
72	40.33	40.33	40.75	1.06	0.94	0.77
96	44.24	43.75	41.61	0.87	0.16	0.07

SEM: Standard error of the mean; Lin: linear effect; Qua: quadratic effect.

^{ab}Means with different letters are significantly different ($P<0.05$).

contrasts with the findings by Cruywagen and Groosen (2004), who observed an increase in weight gain and efficiency of feed utilization in sheep consuming a 66.8% forage diet compounded from wheat straw and alfalfa hay supplemented with 5 and EFE 10 ml kg^{-1} of wheat straw. Similar results were reported by Cruywagen and van Zyl (2008), who added a commercial enzyme complex consisting of cellulase, xylanase and β-mannanase to diets high in forage, and observed increased daily gain and improved feed conversion in sheep. Moreover, in steers on pasture on conditions of humid tropics, feed fermented sugarcane and supplementation with 0, 15 or 30 g animal^{-1} d commercial EFE, produced a positive linear effect on weight gain (Gomez *et al.*, 2011).

Similar positive effects on completion were observed on daily gain and feed conversion by adding doses of 4,000 and 12,500 IU kg^{-1} DM of commercial cellulase and xylanase respectively, to a diet of equal parts wheat straw and concentrate in buffalo (*Bubalus bubalis*) (Malik and Bandla, 2010). Variations in the growth performance of ruminants consuming forages previously treated with fibrolytic enzymes may be due to factors such as specificity and enzyme activity as well as the manner and time of application and the nature of the cell wall of the forage (Eun *et al.*, 2008).

Regarding the influence of EFE levels on ruminal pH, several authors reported an effect similar to that observed in this study *in vivo* tests with sheep (Miller *et al.*, 2008; Pinos-Rodríguez *et al.,* 2008; Gado *et al.*, 2009). Given that in this investigation the pH was maintained in a range of 6.37 to 6.44, the endogenous fibrinolytic microbial activity was maximal, improving the digestion of the cell walls contained in the diet (Colombatto *et al.*, 2007). Moreover, ammonia nitrogen was not affected by the levels of EFE, consistent with other studies with sheep fed enzymes (Baah *et al.*, 2005; Giraldo *et al.*, 2008). However, there was a decrease in $N\text{-}NH_3$ caused by the EFE with respect to the control treatment. This effect has been reported with varying levels of enzymes and fodder (Carreón *et al.*, 2010; Gaafar *et al.*, 2010); because the enzymes increase the digestion of DM and NDF, more energy substrates are released, improving microbial protein synthesis by reducing the concentration of $N\text{-}NH_3$ (Gado *et al.*, 2011).

The percentage of volatile fatty acids was unchanged by the addition of elevated levels of EFE, and a decrease after applying EFE at 5 g kg^{-1} feed, similar to the finding described by Yang *et al.* (2011), who detected decreasing acetic acid concentrations after 12 h of incubation using rice straw treated with EFE.

The results of the *in vivo* digestibility of DM and NDF were similar to those reported in trials conducted *in vitro* using different substrates and levels of EFE (Reddish and Kung, 2007; Almaraz *et al.*, 2010, Gallardo *et al.,* 2010). Our findings *in situ* with regard to the disappearance of the cell walls in the experimental diet coincide with those of Salem *et al.* (2011), who reported an increased disappearance *in situ* of DM and NDF after between 24 and 48 h of incubation for sheep diets containing a high proportion of wheat straw.

The animals' response to utilizing cereal straws is that the exogenous enzymes perform their biocatalytic activity for a short period of time after entering the rumen (Wang *et al.*, 2012). However, poor quality fodder requires more time for the enzymatic action to digest cell wall structures. Moreover, Colombatto *et al.* (2003), report that factors associated with the development of specific conditions, enzymes, substrate quality and handling all affect the efficiency of the EFE.

It is concluded that the addition of high doses of exogenous commercial fibrolytic enzymes did not alter the digestion of dry matter and fibre in diets high of oat straw, and had no positive impact on the performance of producing sheep to finalization.

REFERENCES

Almaraz, I., González, S.S., Pinos-Rodríguez, J.M. and Miranda, L.A. 2010. Effects of exogenous fibrolytic enzymes on *in sacco* and *in vitro* degradation of diets and on growth performance of lambs. *Italian Journal of Animal Science,* 9: 6-10.

Álvarez, G., Pinos-Rodríguez, J.M., Herrera, J.G., García, J.C. and Bárcena, R. 2009. Effects of exogenous fibrolytic enzymes on ruminal digestibility in steers fed high fiber rations. *Livestock Science*, 121: 150-154.

Baah, J., Shelford, J.A., Hristov, A.N., McAllister, T.A. and Cheng, K-J. 2005. Effects of Tween 80 and Fibrolytic Enzymes on Ruminal Fermentation and Digestibility of Feeds in Holstein Cows. *Asian-Australasian Journal of Animal Sciences*, 18: 816-824.

Carreón, L., Pinos-Rodríguez, J.M., Bárcena, R., González, S.S. and Mendoza, G. 2010. Influence of fibrolytic enzymes on ruminal disappearance and fermentation in steers fed diets with short and long particle length of forage. *Italian Journal of Animal Science,* 9: 83-87.

Chunck-Hernández, C., Pérez- Carrillo E., Heredia-Olea E. and Serna-Saldívar. 2011. Sorghum as a multifunctional crop for bioethanol production in Mexico: Technologies, advances and improvement opportunities. *Revista Mexicana de Ingeniería Química*, 10: 529-549.

Colombatto, D., Mould, F.L., Bhat, M.K. and Owen, E. 2003. Use of fibrolytic enzymes to improve the nutritive value of ruminant diets: A biochemical and *in vitro* rumen degradation assessment. *Animal Feed Science and Technology,* 107: 201-109.

Colombatto, D., Mould, F.L., Bhat, M.K. and Owen, E. 2007. Influence of exogenous fibrolytic enzyme level and incubation pH on the *in vitro* ruminal fermentation of alfalfa stems. *Animal Feed Science and Technology,*137: 150-162.

Cruywagen, C.W.and Goosen, L. 2004. Effect of an exogenous fibrolytic enzyme on growth rate, feed intake and feed conversion ratio in growing lambs. *South African Journal of Animal Science,* 34: 71-73.

Cruywagen, C.W. and van Zyl, W.H. 2008. Effects of a fungal enzyme cocktail treatment of high and low forage diets on lamb growth. *Animal Feed Science and Technology,*145: 151-158.

Erwin E.S., Marco G.J. and Emery E. 1961. Volatile fatty acids analysis of blood and rumen fluid by gas chromatography. *Journal of Dairy Science,* 44: 1768-1771.

Eun, J.S. and Beauchemin, K.A. 2007. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. *Animal Feed Science and Technology,* 132: 298-315.

Eun, J.S., ZoBell, D.R., Dschaak, C.M. and Diaz, D.E. 2008. Effect of a fibrolytic enzyme supplementation on growing beef steers. *Proceedings, Western Section, American Society of Animal Science*, 59: 418-420.

Eun, J.-S., ZoBell, D.R., Dschaak, C.M., Diaz, D.E. and Tricarico, J.M. 2009. Case study: Effects of supplementing a fibrolytic feed enzyme on the growth performance and carcass characteristics of beef steers. *The Professional Animal Scientist*, 25: 382-387.

Gaafar, H.M.A., Abdel-Raouf, E.M. and El-Reidy, K.F.A. 2010. Effect of fibrolytic enzyme supplementation and fiber content of total mixed ration on productive performance of lactating buffaloes. *Slovak Journal of Animal Science,* 43: 147-153.

Gado, H.M., Salem, A.Z.M., Robinson, P.H. and Hassan, M. 2009. Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Animal Feed Science and Technology,* 154: 36-46.

Gado H.M., Salem, A.Z.M., Odongo, N.E., Borhami, B.E. 2011. Influence of exogenous enzymes ensiled with orange pulp on digestion and growth performance in lambs. *Animal Feed Science and Technology,* 165: 131-136.

Gallardo, I., Bárcena, R., Pinos-Rodríguez J.M., Cobos, M., Carreón, L. and Ortega, M.E. 2010. Influence of exogenous fibrolytic enzymes on *in vitro* and *in sacco* degradation of forages for ruminants. *Italian Journal of Animal Science,* 9: 34-38.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J., Ramos, S. and Carro, M.D. 2008. Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. *Journal of Animal Science,* 86: 1617-1623.

Gómez, V.A., Mendoza, M.G. and Pinos-Rodríguez J. 2011. Comparison of *in vitro* degradation of elephant grass and sugarcane by exogenous fibrolytic enzymes. *African Journal of Microbiology Research*, 5: 3051-3053.

Herrera, J.G. and García, C. 2010. Bioestadística en Ciencias Veterinarias. Procedimientos de análisis de datos con SAS. ed. Universidad Complutense de Madrid, Madrid, España.

Krueger, N.A. and Adesogan, A.T. 2008. Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. *Animal Feed Science and Technology,* 145: 84-94.

Malik, R. and Bandla, S. 2010. Effect of source and dose of probiotics and exogenous fibrolytic enzymes (EFE) on intake, feed efficiency, and growth of male buffalo (*Bubalus bubalis*) calves. *Tropical Animal Health and Production*, 42: 1263-1269.

McCullough, H. 1967. The determination of ammonia in whole blood by direct colorimetric method. *Clinica Chimica Acta*, 17: 297-304.

Mendoza, M.G.D., Plata, P.F., Ramírez, M.M., Mejía, D.M.A., R.H. Lee, R.H., Bárcena, G.R., 2007. Evaluation of complete feeds for intensive fattening lambs. *Revista Científica de la Facultad de Ciencias Veterinarias de la Universidad del. Zulia*, 15: 66-72.

Meraz-Romero, E., Loera-Corral, O., Mendoza-Martínez, G.D., Meneses-Mayo, M., Cobos- Peralta, M.A., Hernández-Sánchez, D., Angeles-Campos, C. Melgarejo-Velasquez, L.G. and Pinos-Rodríguez, J.M. 2012. Efecto del pH y del líquido ruminal clarificado en la estabilidad de un producto enzimático fibrolítico. *Agrociencia*, 46: 347-358.

Miller, D.R., Elliott, R. and Norton, B.W. 2008. Effects of an exogenous enzyme, Roxazyme® G2 liquid, on digestion and utilisation of barley and sorghum grain-based diets by ewe lambs. *Animal Feed Science and Technology,* 140: 90-109.

NRC. 1985. *Nutrient Requirements of Sheep*, 6th ed. National Academy Press, Washington, DC.

Pinos-Rodríguez, J.M., Moreno, R., González, S.S., Robinson, P.H., Mendoza, G. and Álvarez, G. 2008. Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestibility of total mixed rations fed to lambs. *Animal Feed Science and Technology,* 142: 210-219.

Reddish, M.A. and Kung, L.Jr. 2007. The effect of feeding a dry enzyme mixture with fibrolytic activity on the performance of lactating cows and digestibility of a diet for sheep. *Journal of Dairy Science,* 90: 4724-4729.

Salem, A.Z.M., El-Adawy, M., Gado, H., Camacho, L.M., González, R.M., Alsersy, H. and Borhami, B. 2011. Effects of exogenous enzymes on nutrients digestibility and growth performance in sheep and goats. *Tropical and Subtropical Agroecosystems*, 14: 867-874.

Steel, G.D.R., Torrie, J.H. and Dickey, D.A. 1997. *Principles and Procedures of Statistics. A Biometrical Approach.* The McGraw-Hill Companies, Inc., New York pp. 637.

Tang, S.X., Tayo, G.O., Tan, Z.L., Sun, Z.H., Shen, L.X., Zhou, C.S., Xiao, W.J., Ren, G.P., Han, X.F. and Shen, S.B. 2008. Effects of yeast culture and fibrolytic enzymes supplementation on *in vitro* fermentation characteristics of low-quality cereal straws. *Journal of Animal Science,* 86, 1164-1172.

Van Soest P.J., Robertson J.B. and Lewis B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3597.

van Keulen J. and B.A. Young. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *Journal of Animal Science,* 44: 282-287.

Varlyakov, I., Grigorova, N. and Slavov, T. 2010. Effect of *Hostazym C 100* on growth performance and some hematological and ethological indexes of yearling rams. Bulg. *Journal of Agricultural Science*, 16: 659-664.

Wang, Y., Spratling, B.M., ZoBell, D.R., Wiedmeier, R.D. and McAllister, T.A. 2004. Effect of alkali pre-treatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. *Journal of Animal Science,* 82: 198-208.

Wang, Y., Ramírez, B.J.E., Yanke, L.J., Tsang, A. and McAllister, T.A. 2012. Effect of exogenuos fibrolytic enzyme application on the microbial attachment and digestion of barley straw *In vitro*. *Asian-Australasian Journal of Animal Sciences*, 25: 66-74.

Yang, H.E., Son, Y.S. and Beauchemin, K.A. 2011. Effects of exogenous enzymes on ruminal fermentation and degradability of alfalfa hay and rice straw. *Asian-Australasian Journal of Animal Sciences*, 24: 56-64.

Zinn, R.A. and Ware, R.A. 2002. Fibrolytic enzyme supplementation, a tool for enhancing energy intake in growing-finishing feedlot cattle. Department of Animal Science, University of California, Davis, California, USA, pp. 219-224.

Animal Nutrition and Feed Technology (2013) 13: 363-372

The Effects of a Lignocellulolytic Extract of Fomes sp. EUM1 on the Intake, Digestibility, Feed Efficiency and Growth of Lambs#

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ABSTRACT

Arce-Cervantes, O., Mendoza, G.D., Hernández, P.A., Meneses, M, Torres-Salado, N. and Loera, O. 2013. The effects of a lignocellulolytic extract of Fomes sp. EUM1 on the intake, digestibility, feed efficiency and growth of lambs. Animal Nutrition and Feed Technology, 13: 363-372.

The objective of this experiment was to evaluate the effect of a lignocellulolytic extract (LE), extracted from the thermotolerant basidiomycete *Fomes* sp. EUM1, on the intake, digestibility, feed efficiency, growth and productive performance of lambs. The xylanase and cellulase enzyme activities of the LE were 169 IU g^{-1} and 50 IU g^{-1}, respectively. Eighteen Suffolk crossed lambs (20 ± 0.5 kg body weight) were used for the experiment (58 days). A completely randomized experimental design was used with the following treatments: 0, 60 and 120 mL of LE sprayed per kg of forage 24 h before feeding. Average daily gain and digestibility were improved ($P < 0.05$) with LE supplementation (60 and/or 120 mL). The proportion of butyric acid was reduced with the use of 60 mL of LE ($P < 0.05$). The cellulase activity in the rumen fluid increased linearly ($P < 0.05$) with increasing LE doses. The LE from *Fomes* sp. EUM1 improved the cellulolytic activity in the rumen fluid, thereby increasing the digestibility and body weight gain in lambs.

Key words: Digestibility, Exogenous enzymes, *Fomes* sp, Lambs.

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

Exogenous fibrolytic enzymes have been developed to increase feed digestibility in ruminants (Pinos-Rodríguez *et al.,* 2002; Beauchemin *et al.,* 2003; Colombatto *et al.,* 2003). Commercial products obtained by solid culture fermentation (Loera and Villaseñor, 2006) have been evaluated as enzyme complexes from *Trichoderma longibrachiatum*, *Aspergillus niger* and *Penicillium funiculosum* strains (Rojo *et al.,* 2005; Eun and Beauchemin, 2007; Giraldo *et al*., 2008). In addition, the treatment of forages using enzyme supplements does not pose a toxic hazard and is an effective method for improving feed quality (Krueger *et al.,* 2008).

White-rot fungi (recognized as safe) synthesize lignocellulolytic complexes that promote the degradation of cell wall structural carbohydrates (Papinutti *et al*., 2008; Elisashvili *et al.,* 2009). These enzymes could be used to improve the digestibility of lignocellulosic substrates fed to ruminants (Agosin *et al.,* 1985; Rodrigues *et al., 2*008; Peláez-Acero *et al.,* 2011).

There is evidence that extracts obtained from lignocellulolytic white-rot fungi may modify the degradability of agricultural by-products. Thus, the thermotolerant strain *Fomes* sp. EUM1 was identified by Ordaz *et al*. (2012), and an *in vitro* incubation of sorghum straw with extracts from this strain were shown to double the microbial adherence (Villegas-Castañeda *et al.,* 2010); in addition, an LE from this fungus also increased the *in vitro* digestibility of corn stover. However, this LE has been not evaluated *in vivo*. Therefore, the aim of this study was to obtain an enzyme extract of *Fomes* sp. EUM1 in solid culture and to evaluate its effect on the intake, digestibility, feed efficiency and growth in lambs.

MATERIALS AND METHODS

Production of lignocellulolytic extracts and determination of enzyme activities

The fungus *Fomes* sp. EUM1 was acquired from the fungal collection from the Universidad Autónoma Metropolitana, Mexico. The enzymatic extract was obtained by solid-state cultivation using corn stover (2.4 g) with wheat bran (0.6 g) as the substrate. Corn stover was sieved to a selected particle size ($>0.81, <4.6$ mm), as recommended by Membrillo *et al*. (2008). The cultures were incubated at 35°C for 6 days with an initial moisture of 80%. Extracts were obtained by adding 100 mL of distilled water (pH 6.5) under constant stirring in an ice bath for 20 min. The extracts were then passed through Whatman No. 1 filters and centrifuged (15.008 *g*, 4°C, 15 min). The supernatant was recovered as the lignocellulolytic enzymatic extract (LE), which was used for enzymatic determinations (Da Silva *et al.,* 2005).

The activity of xylanases and cellulases was determined by the reducing sugar method (Miller *et al.,* 1960). For xylanases, 0.5% xylan from Birchwood (Sigma-Aldrich) prepared by diluted with 50 mM sodium citrate buffer (pH 5.3) (Loera and

Cordova, 2003) was used as a substrate. For cellulases, 1% carboxymethylcellulose (Sigma-Aldrich) dissolved in citrate buffer (50 mM, pH 5) was used as the substrate. Standard curves were prepared with xylose for xylanases and glucose for cellulases. The absorbance was read at 640 nm using a Beckman DU649 spectrophotometer. Enzyme activities were reported as International Units (IU) per gram of initial dry matter (IU g DM^{-1}), where one IU is the amount of enzyme that releases 1 μmol of reducing sugar (xylose or glucose) per minute.

Laccase activity was determined using ABTS (2, 2-azinobis-3-ethylbenzthiazolinesulphonic) at 0.1 M, diluted in citrate buffer (50 mM, pH 5). The absorbance was read at 420 nm every 10 s for 1.50 min (Bourbonnais *et al.,* 1997). The laccase activity is reported as IU g DM^{-1}, where one IU is the amount of enzyme that releases 1 μmol of oxidized ABTS per minute under the reaction conditions.

Experiment with lambs

The experiment was conducted according to regulations of the Animal Protection Law enacted by the Estado de Mexico. The experiment was performed in Jilotepec, Mexico, using 18 Suffolk crossed lambs with an initial weight of 20 kg$\pm$0.5 kg and placed in individual pens (2 m^2) with a concrete floor. The lambs were vaccinated 10 days prior to the experiment (Bacterin Polivalent), and after adaptation to the diet of 21 days, they were weighed (initial weight of the experiment) and dewormed (Ivomec). Lambs had free access to feed (Table 1) at 09:00 and 17:00 (ensuring 100 g orts per kg of the amount fed daily), and water was provided *ad libitum*. The composition of the basal diet is presented in Table 1. The experimental design was completely randomized, and the treatments were 0, 60 and 120 mL of LE per kg of dry corn stover. Doses were added to 500 mL of distilled water and sprayed onto the feed 24 h before allocating the food (Alvarez *et al.,* 2009).

Table 1. Ingredients and chemical composition of the basal diet (g kg^{-1} dry matter)

Ingredients	LE† (mL kg^{-1} DM)		
	0	60	120
Corn stover	300	300	300
Alfalfa hay	300	300	300
Concentrate‡	400	400	400
Chemical composition (g kg^{-1} DM)			
Dry matter	928	929	930
Crude protein	145	142	141
Neutral detergent fiber	570	586	585
Acid detergent fiber	339	342	333

†LE, lignocellulolytic enzymatic extract; ‡Union Tepexpan (México) 12% crude protein

The experiment lasted 58 days. Lambs were weighed at the beginning of the experiment and every 14 days. Faecal grab samples were collected from lambs for 4 days beginning on day 30 to estimate the digestibility using acid insoluble ash as internal marker (Keulen *et al.,* 1977). Feed was analysed for dry matter (ID 934.01), organic matter (ID 942.05) and total nitrogen (ID 984.13) contents following the techniques of the AOAC (1990); neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analysed according to Van Soest *et al.* (1999) using an Ankom fiber analyser (Ankom Technology Corp., NY). Faeces were analysed for DN, NDF and ADF using the same procedures used for the feed analysis.

Sampling of rumen fluid and determination of volatile fatty acids and NH_3-N

On day 58 of the experiment, an esophageal probe was used to collect rumen fluid 4 h post-prandial. Fluid was filtered through 4 layers of cheesecloth, and the pH was measured immediately. Ruminal fluid was then subdivided into two subsamples: the first was used for VFA and ammonia-N determination after preparation with metaphosphoric acid (5%) at a rumen fluid:acid ratio of 4:1; the second subsample was used to measure enzymatic activity and was frozen (-10°C) until analysis.

Subsamples prepared with metaphosphoric acid were centrifuged at 18,000 *g* for 20 min. For VFA, 1 mL was injected into a gas chromatograph (Perkin Elmer, model Clarus 500), as described by Erwin *et al.* (1961). The ammonia-N concentration was determined by spectrophotometry (Varian, model Cary IE) according to the procedure of McCullough (1967).

Enzyme activity in rumen fluid

Rumen fluid samples were thawed and placed in a sonicator for 10 min, followed by 5 min at rest. The sonication was repeated, and the fluid was then stirred manually. The sonicated sample was centrifuged for 15 min at 15,000 *g* at 4°C (Hristov *et al.,* 2008). The obtained extract was used to evaluate the enzymatic activity using the aforementioned procedures.

Statistical analysis

Data were analysed using the GLM procedure of SAS version 9 (SAS, 2002). The initial weight of the lambs was used as a covariate for the intake and feed conversion data. The response to the enzyme level was tested for linear and quadratic effects (Steel *et al.,* 1997), and the means were compared using the Tukey test.

RESULTS AND DISCUSSION

Enzymatic activities of the extracts

The LE obtained from the cultures of *Fomes* sp. EUM1 had a xylanase activity of 169 IU g DM^{-1}, cellulase activity of 50 IU g DM^{-1} and laccase activity of 4 IU g DM^{1}.

Certain researchers have noted that the amendment of ruminant diets using the enzymes produced by white-rot fungi could have beneficial effects (Graminha *et al.*, 2008; D'Agostini *et al.*, 2011). However, evaluations using LE from these basidiomycetes remain rare, and the enzymatic activity varies depending on the type of organism, particularly in commercial products. When comparing the fibrolytic activity from *Fomes* sp. EUM1 in this trial with that of Fibrozyme®, the cellulolytic activity was similar, although the xylanolytic content is 24% lower than in the commercial product (Ramirez *et al.,* 2005).

In this experiment, all LEs were incorporated to the corn stover because the extent of fiber degradation increases when the enzymes are incubated with the forage (Krueger *et al.*, 2008). Yang *et al*. (2004) reported the need to treat the plant fibers under aerobic conditions to promote the formation of hydroxyl radicals, which facilitates their hydrolysis; this effect is directly related to the source of the enzymatic extracts. Moreover, the digestibility results suggest that the addition of exogenous enzyme helped to remove phenolic barriers that limit the microbial digestion of the cell wall (Wang *et al.,* 2004) and perhaps improved the colonization of the food particles by ruminal bacteria (Wang and McAllister, 2002). It has been suggested that greater benefits would be obtained using a combination of fibrolytic enzymes (Eun and Beauchemin 2007); in this context, Wang *et al*. (2004) recommended that preparations should include esterase activity. These combined effects may be attained using the LE from *Fomes* sp. EUM1, which is capable of producing several types of enzymes (Marquez-Araque *et al.*, 2007).

Lamb performance and digestibility

There were no significant effects ($P>0.05$) of the enzyme dose on the intake of the various evaluated fractions (Table 2) or the feed conversion. The average daily gain was improved ($P<0.05$) relative to the control group but was similar between the two doses of LE (Table 3). The digestibility of DM, NDF and ADF were improved with the intermediate dose of LE (quadratic effect, $P<0.05$; Table 3).

Table 2. Intake treatment for lambs receiving doses of exogenous crude enzymes (g day^{-1})

Component	†LE (mL kg^{-1} DM)			SEM	P-value	
	0	60	120		Linear	Quadratic
Dry matter	1289	1367	1397	96	0.44	0.83
Organic matter	1066	1141	1166	80	0.39	0.80
NDF	731	790	798	55	0.40	0.70
ADF	426	451	458	32	0.48	0.80
Nitrogen	198	197	187	14	0.60	0.78

†LE, lignocellulolytic enzymatic enzyme; NDF, neutral detergent fiber; ADF, acid detergent fiber; SEM, standard error of the mean.

Previous studies have reported that enzymatic treatment did not affect nutrient intake (Cruywagen and Goosen 2004; Alvarez *et al.*, 2009; Giraldo *et al.*, 2008); however, intake can be modified depending on the forage quality and the type of enzymes that are used (Pinos-Rodríguez *et al.*, 2002; Bernard *et al.,* 2010). Nevertheless, Cruywagen and van Zyl (2008) reported that consumption was affected by the forage: concentrate ratio but not by the addition of exogenous enzymes.

In this study, the inclusion of extracts containing exogenous enzymes improved the daily weight gain by up to 15% without affecting the feed conversion (Table 3). In lambs fed a diet high in concentrates, Cruygawen and Goosen (2004) found that different doses of commercial xylanases and cellulases from *A. terreus* significantly increased the weight gain by up to 32% and improved the feed conversion by up to 25%, with a maximum dose of 40% concentrate, in lambs fed wheat straw and alfalfa hay. The results from multiple experiments have indicated that weight gain can be improved with enzyme amendments, although the response will vary as a function of the selected enzymes, doses and substrate (Pinos-Rodríguez *et al.*, 2002), and the response can be associated with the improvements in digestibility.

Table 3. Performance of lambs fed high forage (60:40) with enzymatic treatment

Item	†LE (mL kg^{-1} DM)			SEM	P-value	
	0	60	120		Linear	Quadratic
Initial weight (kg)	20.0	19.9	20.0	2.06	-	-
Final weight (kg)	32.3	34.1	34.1	2.34	0.58	0.75
ADG (g d^{-1})	211[d]	244[c]	243[c]	0.01	0.08	0.25
Feed conversion	6.2	5.6	5.8	0.39	0.52	0.44
Digestibility (%)						
Dry matter	48.9[b]	58.5[a]	48.8[b]	3.4	0.97	0.03
Organic matter	38.5	49.9	38.3	4.1	0.96	0.37
NDF	42.9[b]	57.8[a]	39.0[b]	3.3	0.41	0.009
ADF	32.9[b]	48.0[a]	18.4[b]	4.2	0.02	0.006
Nitrogen	54.4	60.6	50.3	3.6	0.42	0.07

†LE, lignocellulolytic enzymatic extract; ADG, average dairy gain; NDF, neutral detergent fiber; ADF, acid detergent fiber; SEM, standard error of the mean.

[ab]Means within each row with different superscripts are significantly different ($P<0.05$).

[cd]Means within each row with different superscripts are significantly different ($P<0.10$).

In vitro tests have shown that extracts from *Fomes* sp. EUM1 did not improve *in vitro* digestibility of sorghum straw (Villegas-Castañeda *et al.*, 2010), whereas enzymes from *Pleurotus ostreatus* improved the *in vitro* digestibility of fiber (Peláez-Acero *et al.*, 2011). Another study demonstrated that the use of enzymatic extracts from *Trametes versicolor, Bjerkandera adusta* and *Fomes fomentarius* resulted in enhanced *in vitro* degradation of wheat straw NDF with a synergistic effect between the enzymes (Rodrigues *et al.*, 2008). It is possible to speculate that the response is a function of the

potentially digestible fraction of the forage and that a higher NDF fraction corresponds to a stronger response to the addition of diverse exogenous enzymes. Depending on the magnitude of the effect of enzymes on the NDF digestibility, enzyme additions can enhance the weight gain and, in certain cases, increase the intake as a consequence of a reduction in the rumen fill.

Ruminal variables

The pH and ammonia N and VFA concentrations in the ruminal fluid were not affected by the enzymatic extracts (Table 4); however, the proportion of butyric acid was reduced with the dose of 60 mL kg^{-1} DM (quadratic effect, $P<0.05$). The total cellulolytic activity in the rumen fluid presented a linear increase ($P<0.05$) as the dose of LE increased.

Numerous experiments have shown only small effects of the addition of fibrolytic enzymes on VFA concentrations and fermentation patterns (Alvarez *et al.*, 2009; Muhamad and Suwandyastuti, 2005). However, it has also been demonstrated that responses vary according to the substrate and enzyme that are used (Beauchemin *et al.,* 2003; Pinos-Rodriguez *et al.,* 2002). In this study, the levels of butyric acid were reduced with the intermediate dosage level; similar results were observed by Gonzalez-Garcia *et al*. (2009) with goats fed a diet with 70% forage amended with commercial cellulases (Promote). The reduction in the proportion of butyrate may be explained by changes in the population of *Butyriovibrio fibrisolvens* or by a stimulation of the Holotricha protozoa that synthesize butyrate, such as *Isotricha ruminantum* and *Dasytricha prostoma*. The released of excessive levels of soluble sugars via the activity of exogenous enzymes may have stimulated the population of these ciliates (Luther *et al.,* 1966).

Table 4. Fermentation pattern and enzyme activities of lambs fed high forage (60: 40) and amended with various doses of exogenous crude enzyme preparations

Item	†LE (mL kg^{-1} DM)			SEM	P-value	
	0	60	120		Linear	Quadratic
pH	6.5	6.6	6.5	0.18	0.91	0.86
NH_3	8.7	9.6	7.7	1.57	0.67	0.48
VFA (mol/100 mol)						
Acetate	64.8	66.0	65.2	0.72	0.76	0.29
Propionate	21.1	21.9	20.4	0.85	0.54	0.29
Butyrate	14.0[a]	12.2[b]	14.5[a]	0.50	0.56	0.004
Total VFA	51.03	52.26	56.80	6.27	0.98	0.86
Enzyme activity (IU mL^{-1})						
Xylanases	215.1	202.6	225.5	30.36	0.81	0.64
Cellulase	6.4[b]	11.5[a]	9.7[a]	0.88	0.01	0.006

†LE, lignocellulolytic enzymatic extract; SEM, standard error of the mean.

[ab]Means within each row with different superscripts are significantly different ($P<0.05$).

The total cellulolytic enzymatic activity in the rumen fluid of lambs was increased by 78 and 50% with the lower and higher doses of extract, respectively. Hristov *et al.* (2008) observed a 60% increase in the cellulase and xylanase activities in ruminal contents relative to controls when exogenous enzymes were added to feed in lactating cows. As the dose of exogenous enzyme is increased, the cellulolytic activity in the rumen increases linearly at up to 22% relative to the control (Hristov *et al.,* 2000) with the highest activity observed at 4 hours post prandial (Hristov *et al.*, 2008). The higher digestibility with the intermediate dose was associated with the cellulolytic activity in the rumen fluid, although the changes in the butyrate levels did not affect the growth performance among the treatments with LE.

CONCLUSION

Crude enzymatic extracts of the thermotolerant basidiomycete *Fomes* sp. EUM1 improved the dry matter digestibility and increased the NDF and ADF contents with an intermediate dose, increasing the cellulolytic activity in the rumen fluid and manifesting a higher weight gain in lambs with the extract enzyme.

ACKNOWLEDGEMENTS

The authors thank the National Council of Science and Technology (CONACyT) for the scholarship granted to Arce-Cervantes (Grant 198319) and are grateful for the financial support provided by the Universidad Autonóma Metropolitana and the RED-Promep.

REFERENCES

Agosin, E., Monties, B. and Odier, E. 1985. Structural changes in wheat straw components during decay by lignin-degrading white-rot fungi in relation to improvement of digestibility for ruminants. *Journal of Science Food and Agriculture*, 36: 925-935.

Alvarez, G., Pinos-Rodríguez, J.M., Herrera, J.G., García, J.C., González, S.S. and Bárcena, R. 2009. Effects of exogenous fibrolytic enzymes on ruminal digestibility in steers fed high fiber rations. *Livestock Science,* 121: 150-154.

AOAC. 1990. *Official Methods of Analysis,* 12th ed. Association of Official Analytical Chemists, Washington, DC, USA.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve fed utilization by ruminants. *Journal of Animal Science,* 81: E37-E47.

Bernard, J.K., Castro, J.J., Mullis, N.A., Adesogan, A.T., West, J.W. and Morantes, G. 2010. Effect of feeding alfalfa hay or Tifton 88 Bermuda grass haylage with or without a cellulose enzyme on performance of Holstein cows. *Journal of Dairy Science,* 93: 5280-5285.

Bourbonnais R., Pice M.G., Freiermuth B., Bodie E. and Borneman S. 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Applied Environmental Microbiology*, 63 (Suppl. 12): 4627-4632.

Colombatto, D., Fergus, L.M., Mahalingeshwara, K.B. and Owen, E. 2003. Use of fibrolytic enzymes to improve the nutritive value of ruminants diets: A biochemical and *in vitro* rumen degradation assessment. *Animal Feed Science and Technology,* 107: 201-209.

Cruywagen, C.W. and Goosen, L. 2004. Effect of an exogenous fibrolytic enzyme on growth rate, feed intake and feed conversion ratio in growing lambs. *South African Journal of Animal Science,* 34 (Suppl. 2): 71-73.

Cruywagen, C.W. and van Zyl, W.H. 2008. Effects of a fungal enzyme cocktail treatment of high and low forage diets on lamb growth. *Animal Feed Science and Technology,* 145: 151-158.

Da Silva, R., Lago, E.S., Merheb, C.W., Macchione, M.M., Kun, P.Y. and Gomes, E. 2005. Production of xylanase and CMCase on solid state fermentation in different residues by *Thermoascus aurantiacus* Miehe. *Brazilian Journal of Microbiology,* 36: 235-241.

D'Agostini, E.C., Mantovani, T.R.D., do Valle, J.S., Paccola-Meirelles, L.D., Colauto, N.B. and Linde, G.A. 2011. Low carbon/nitrogen ratio increases laccase production from basidiomycetes in solid substrate cultivation. *Scientia Agricola,* 68: 295-300.

Elisashvili, V., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Khardziani, T. and Agathos, S.N. 2009. Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the forests of Georgia. *World Journal of Microbiology Biotechnology,* 25: 331-339.

Erwin, E.S., Marco, G.T. and Emery, E.M. 1961. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. *Journal of Dairy. Science,* 44: 1768-1771.

Eun, J.S. and Beauchemin, K.A. 2007. Enhancing *in vitro* degradation of alfalfa hay and corn silage using feed enzymes. *Journal of Dairy. Science*, 90: 2839-2851.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J., Ramos, S. and Carro, M.D. 2008. Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. *Journal of Animal Science,* 86: 1617-1623.

Gonzalez-García, E., Albanell, E., Caja, G. and Casals, R. 2009. *In vitro* fermentative characteristics of ruminant diets supplemented with fibrolytic enzymes and ranges of optimal endo -β1, 4-glucanase activity. *Journal of Animal Physiology and Animal Nutrition,* 94: 250-263.

Graminha, E.B.N., Gonçalves, A.Z.L., Pirota, R.D.P.B., Balsalobre, M.A.A., Da Silva, R. and Gomes, E. 2008. Enzyme production by solid-state fermentation: Application to animal nutrition. *Animal Feed Science and Technology,* 144: 1-22.

Hristov, A.N., McAllister, T.A. and Cheng, K.J. 2000. Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: effects on nutrient digestion in cattle fed a barley grain diet. *Journal of Animal Science,* 78: 477-487.

Hristov, A.N., Basel, C.E., Melgar, A., Foley, A.E., Ropp, J.K., Hunt, C.W. and Tricarico, J.M. 2008. Effect of exogenous polysaccharide-degrading enzyme preparations on ruminal fermentation and digestibility of nutrients in dairy cows. *Animal Feed Science and Technology,* 145: 182-193.

Keulen, J.V. and Young, B.A. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *Journal of Animal Science,* 44: 282-287.

Krueger, N.A., Adesogan, A.T., Staples, C.R., Krueger, W.K., Dean, D.B. and Litell, R.C. 2008. The potential to increase digestibility of tropical grasses with a fungal, ferulic acid esterease enzyme preparation. *Animal Feed Science and Technology,* 145: 95-108.

Loera, O. and Córdova, J. 2003. Improvement of xylanase production by a parasexual cross between *Aspergillus niger* strains. *Brazilian Archives of Biology and Technology,* 46 (Suppl. 2): 177-181.

Loera, O. and Villaseñor, F. 2006. Xylanases. In: *Advances in Agricultural and Food Biotechnology* (Eds. R.G. Guevara and I. Torres), Research Signpost Editorial, Kerala, India, pp. 305-322.

Luther, R., Trenkle, A. and Burroughs, W. 1966. Influence of rumen protozoa on volatile acid production and ration digestibility in lambs. *Journal of Animal Science,* 25: 1116-1122.

Marquez-Araque, A.T., Mendoza-Martínez, G.D., González, S., Buntinx, S. and Loera, O. 2007. Fibrolytic activity of enzymes produced by *Trametes* sp. EUM1, *Pleurotus ostreatus* IE8 and *Aspergillus niger* AD96.4 in solid fermentation. *Interciencia*, 32: 780-785.

Membrillo, I., Sánchez, C., Meneses, M., Favela, E. and Loera, O. 2008. Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains. *Bioresource Technology,* 99: 7842-7847.

Miller, G.L., Blum, R., Glennon, W.E. and Burton, A.L. 1960. Measurement of carboxymethylcellulase activity. *Analytical Biochemistry*. 2: 127-132.

Muhamad, B. and Suwandyastuti, S.N.O. 2005. The improving quality of concentrate diet with fibrolytic enzyme and its effect on rumen metabolism and blood parameter of fattening Holstein male. *Animal Production,* 7: 127-134.

Ordaz, A., Favela, E., Meneses, M., Mendoza, G. and Loera, O. 2012. Hyphal morphology modification in the thermal adaptation by the white rot fungus *Fomes* sp. EUM1. *Journal of Basic Microbiology,* 52: 167-174.

Papinutti, L., Dimitriu, P. and Forchiassin, F. 2008. Stabilization studies of *Fomes sclerodermeus* laccases. *Bioresource Technology,* 99: 419-424.

Peláez-Acero, A., Meneses-Mayo, M., Miranda-Romero, L.A., Ayala-Martínez, M., Crosby-Galván, M.M., Loera-Corral, O. and Mejías-Rivas, M.D. 2011. Fibrolytic enzymes produced by solid-state fermentation to improve sugar cane silage. *Agrociencia*, 45: 675-685.

Pinos-Rodríguez, J.M., González, S.S., Mendoza, G.D., Bárcena, R., Cobos, M.A., Hernández, A. and Ortega, M.E. 2002. Effect of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. *Journal of Animal Science,* 80: 3016-3020.

Ramirez, C.L., Aranda, E.I., Mendoza, G.D., Landois, P.L., Miranda, R.L.A. and Crosby, G.M.M. 2005. Caracterización de productos fibrolíticos comerciales utilizados en la alimentación de rumiantes. *Veterinaria México,* 36: 1-9.

Rodrigues, M.A.M., Pinto, P., Bezerra, R.M.F., Dias, A.A., Guedes, C.V.M., Cardoso, V.M.G., Cone, J.W., Ferreira, L.M.M., Colaço, J. and Sequeira, C.A. 2008. Effect of enzymes extracts isolated from white-rot fungi on chemical composition and *in vitro* digestibility of wheat straw. *Animal Feed Science and Technology,* 141: 326-338.

Rojo, R., Mendoza, G.D., González, S.S., Landois, L., Bárcena, R. and Crosby, M.M. 2005. Effects of exogenous amylases from *Bacillus lichenoformis* and *Aspergillus niger* on ruminal starch digestion and lamb performance. *Animal Feed Science and Technology,* 123-124: 655-665.

SAS. 2002. *SAS User's Guide: Statistics*. Version 8.02. Cary, NC, SAS Inst.

Steel, G.D.R., Torrie, J.H. and Dickey, D.A. 1997. *Principles and Procedures of Statistics. A Biometrical Approach.* The McGraw-Hill Companies, Inc., New York, pp. 363-365.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fibre, neutral detergent fibre, and nonstarch carbohydrates in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3597.

Villegas-Castañeda, M., Meneses-Mayo, M., Miranda-Romero, L.A. and Loera-Corral, O. 2010. Producción de gas *in vitro* y desaparición de la matera seca del cultivo sólido con hongos ligninolíticos. *Agrociencia*, 44: 917-929.

Wang, Y. and McAllister, T.A. 2002. Investigation of exogenous fibrolityc enzymes activity on barley straw using *in vitro* incubation. *Journal of Animal Science,* 80: (Suppl. 1) 316.

Wang, Y., Spratling, B.M., ZoBell, R.D., Wiedmeier, R.D. and McAllister, T.A. 2004. Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. *Journal of Animal Science,* 82: 198-208.

Yang, W., Liu, J., Wang, W., Zhang, Y. and Gao, P. 2004. Function of a low molecular peptide generated by cellulolytic fungi for the degradation of native cellulose. *Biotechnology Letters,* 26: 1799-1802.

Animal Nutrition and Feed Technology (2013) 13: 373-380

Effect of adding *Salix babylonica* Extracts and Exogenous Enzymes to Basal Diets on the Meat Quality of Growing Suffolk Lambs#

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ABSTRACT

Cayetano, J.A., Salem, A.Z.M., Mariezcurrena, B.M.A., Rojo, R., Cerrillo-Soto, M.A., Gado, H. and Camacho, L.M. 2013. Effect of adding Salix babylonica extracts and exogenous enzymes to basal diets on the meat quality of growing Suffolk lambs. Animal Nutrition and Feed Technology, 13: 373-380.

It was evaluated the effect of adding *Salix babylonica* L. extracts and exogenous enzymes in combination or individually on meat quality in growing Suffolk lambs. Animals were divided into 4 groups of 4 animals each in a completely randomized design (CRD). Treatments were: (i) Control: basal diet of concentrate (30%) mixture and corn silage roughage (70%); (ii) EZE (exogenous enzymes): basal diet plus 10 g of enzyme (Zado®); (iii) SB (*Salix babylonica*): basal diet plus 30 ml of *S babylonica* extracts, and (iv) EZESB (exogenous enzymes + *Salix babylonica*): basal diet plus 10 g enzyme and 30 ml of *S. Babylonica* extracts. Lambs were housed in individual cages for 60 days. Extracts were dosed orally while EZE was mixed with concentrate. At the end of the trial, lambs were slaughtered and *Longissimus dorsi* samples were analysed. Samples were analysed for CP, CF, ash and DM. Meat quality parameters included color, pH, carcass temperature and kidney fat. No significant differences for live weight, chemical composition, as well as hot carcass weight and cold, initial and final temperature and kideny fat. Meat lightness (variable L *) and pHf were improved ($P<0.05$) with EZESB treatment compared to the other three treatments, and the most optimal pHf four treatments being the most acidic. Lambs fed SB or EZE were not different from the control. In conclusion, a combined administration of EZESB to the basal diet improves meat quality by reducing the pH and increasing its lightness when compared to either EZE or SB, individually.

Key words: Exogenous enzymes, Lambs, Meat quality characteristics, *Salix babylonica* extracts

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

Globally, it is recognized that the cost of feeding livestock accounts for over 50% of total production costs, thus there is a constant search for alternative feeding methods that will allow for optimum growth and performance with minimum economic investment. Some of the alternative production methods developed to date include the use of pre and probiotics, ionophores, exogenous enzymes and even local fodder shrubs and trees. Use of foliage from local resources has helped in some areas to reduce costs of production considering that most of these trees grow naturally and there are virtually no management costs associated with their production. In Mexico, the search for these alternatives production/feeding methods has become relevant. Studies performed in Egypt have reported some profitable alternative methods preparing animal feeds (Salem *et al.*, 2006, 2007, 2010). Research conducted with local trees and shrubs and extracts from the same species as additives indicated they represent an important potential in feeding ruminants in arid and semi-arid regions of Northern Egypt.

Addition of fodder tree and shrub extracts to the basal diet of sheep have resulted in positive effects in performance, nonetheless, the addition of exogenous enzymes might prove further improvements in sheep nutrition practices. Utilization of tree/shrub extracts have promoted fiber digestion (non-starch polysaccharides), reduced viscosity of the bolus, increased the bioavailability of nutrients, inhibited the growth of pathogens bacteria and enhanced the growth of probiotic intestinal flora by the hydrolysis of non-starch which allow a better use of the energy value of cereals. Moreover, plant extracts tend to reduce losses and excretion of certain compounds such as phosphorus and nitrogen, reducing negative environmental impacts. Furthermor, they prevent diarrhea and digestive disorders and as a result improves growth performance, reproductive, and animal immune system. Gado *et al.* (2007) indicated that supplementing dairy and beef cattle with exogenous improved fiber degradation, such information was further supported in lambs through *in vitro* and *in vivo* studies (Gado and Salem, 2008). Meat quality evaluation in sheep supplemented with extracts and exogenous enzymes ought to be performed. Bianchi (2007) mentioned that an important attribute for the consumer while buying meat was color, however, other features such as pH, water holding capacity, flavour, smell and fatty acid concentration are also considered, the later being an important issue in human health due to cardiovascular disease concerns. Previous work have indicated positive impacts while using *Salix babylonica* extracts on productive parameters, whereas favourable properties have also been determined by addition of exogenous enzymes (Zado®), thus, this study was conducted to evaluate the meat quality response of these two additives added individually or in combination hoping for positive effects on meat quality of Suffolk lambs.

MATERIALS AND METHODS

The research was conducted at the premises of the zoo technical post at the Faculty of Veterinary Medicine of the Autonomous University of the State of Mexico.

Extract preparations and quality meat evaluations conducted at the Food Science Laboratory of Animal Nutrition of the Faculty.

Preparation of extracts

The extraction of tree/shrub (SB) was conducted according to Salem (2006), where leaves of *S. babylonica* were collected randomly from several young and mature trees during Summer. The fresh leaves were chopped into 1-2 cm lengths and immediately extracted with a prepared solvent mixture (1 g leaf/8 ml). The extraction mixture contained 10 ml methanol (99.8%, analytical grade, Fermont®, Monterrey, Mexico), 10 ml ethanol (99% analytical grade, Fermont®, Monterrey, Mexico) and 80ml distilled water. The chopped leaf materials were individually soaked and incubated in the solvent mixture at laboratory room temperature at 25-30°C for 48 to 72 hrs in closed black/dark 500 ml bottles. After incubation, jars were heated at 39°C for 1 h, and then immediately filtered after which the filtrates were collected and stored at 4°C for further use.

Animals and treatments

Sixteen growing male lambs (4 animals in each treatment) (28 kg BW) and from 7 to 8 months were used. Animals were fed a basal diet consisting of 70% corn silage and 30% commercial concentrate (Purina®) for sixty days. Lambs were provided throughout the study with feed and drinking water *ad libitum.*

Treatments were: (i) Control: basal diet of concentrate (30%) mixture and corn silage roughage (70%); (ii) EZE (exogenous enzymes): basal diet plus 10 g of enzyme (Zado®; commercial product from El Cairo, Egypt). The enzyme treatment was prepared by mixing 10 g of enzyme complex with 200 g concentrate mixture fed to each animal; (iii) SB (*Salix babylonica*): basal diet plus 30 ml of *S. babilonica* extracts; lambs were drenched with the extract directly in the mouth, and (iv) EZESB (exogenous enzymes + *Salix babylonica*): basal diet plus 10 g enzyme and 30 ml of *S. Babylonica* extracts. *Th*e experiment was performed during 60 days.

Measurements and instrumentation

Temperature of hot carcass and pH was recorded on carcass immediately after slaughter and at 24 hours post-mortem. Samples from the *Longissimus dorsi* muscle were collected from 12, 13, 14, 15th ribs at 24 hours post-mortem and stored in a freezer for later analysis.

pH Measurements: The determination of pH and temperature of carcass were performed using a pH meter that was equipped with a penetration electrode (HANNA HI99163). Measurements were taken on the *Longissimus dorsi* muscle (at the last rib). pH was measured by electrode penetration at 45 min after slaughter (time taken to reach carcass) and at 24 h.

Chemical composition analysis

Samples stored in the freezer, which were tere taken from the ribeye area of the 12th and 13th rib, were thawed at 4°C for 24 hours and immediately analysed for moisture content, crude protein, ether extract and ash, according to the AOAC (1990).

Kidney fat scoring

The amount of fat covering the kidneys were scored on a scale of 1 to 4, where 1 was for Kidneys that were completely uncovered 2: Kidneys with large a uncovered window, 3: Kidneys with a small uncovered window and 4 for kidneys that were completely covered in fat. The methodology used for scoring renal fat coverage was according to Delfa *et al*. (1995).

Colour scoring

To measure the colour of meat from the various treatments, frozen meat chops were first thawed at 3°C, measurements were performed using a Minolta Chroma Meter CR-measuring head 400, calibration plate CR.A43, viewing angle 20°, according to Brewer (1999) and Honikel (1998).

Statistical analysis

Data were analysed according to a completely randomized design, with 4 treatments and 4 replications for each treatment. The statistical model used was: $Y_{ijk} = m + t_iTi + E_{ijk}$, where m is the mean, T_i as the treatment effect and E_{ijk} as the residual error effect. Data was analysed using the GLM procedure of SAS (2002). Tukey's test was used for mean comparisons (Steel and Torrie, 1980).

RESULTS

In Table 1, initial pH values (pHi) were not affected by dietary treatments ($P > 0.05$). However, for the final pH (pHf), animals offered the SB had lower values ($P < 0.05$) compared to those registered in lambs fed the control and the EZE treatments. Initial and final meat temperature values were not different among treatments ($P > 0.05$).

Table 1. The effect of adding *Salix babylonica* extracts (SB), exogenous enzymes (EZE) or the mixture of the two (EZE-SB) on pH and the carcass temperature in sheep

Variables	Treatments				LSD	P
	Control	EZE	SB	EZE-SB		
pHi	6.87	6.83	6.80	6.73	0.300	0.572
pHf	6.51[a]	6.53[a]	6.46[ab]	6.25[b]	0.226	0.010
Ti	20.27	21.36	20.55	20.82	2.226	0.556
Tf	8.82	8.23	8.00	8.02	1.206	0.174

pHi: pH initial, pHf: pH final, Ti: initial temperature, Tf: final temperature, EZE: exogenous enzymes, SB: *Salix babylonica*, EZE-SB: exogenous enzymes + *Salix babylonica*

The meat production parameters are shown in Table 2. No significant differences were registered among treatments in all the studied variables. Nonetheless, some numerical trends in BLW, HCW, CCW and KF were observed. Highest record variables regarding weight were registered in animals fed EZE treatment, followed by EZE-SB, SB and Control. Kidney fat was slightly increased in lambs fed EZE than other treatments. This being one of the parameters that give the conformation pattern of sheep body fat, thus supporting the fact that EZE-SB can be considered an suitable treatment.

Table 2. The effect of adding *Salix babylonica* extracts (SB), exogenous enzymes (EZE) or the mixture of the two (EZE-SB) on some production parameters in sheep

Variables	Treatments				LSD	P value
	Control	EZE	SB	EZE-SB		
BLW	34.47	40.13	36.85	37.37	15.656	0.775
HCW	16.30	18.96	16.00	17.47	9.145	0.783
CCW	15.92	18.50	15.50	17.17	9.389	0.791
KF	3.50	3.66	3.50	3.25	1.234	0.797

BLW: Body live weight, HCW: hot carcass weight, CCW: cold carcass weight, KF: kidney fat, EZE: exogenous enzymes, SB: *Salix babylonica*, EZE-SB: exogenous enzymes + *Salix babylonica*.

Meat chemical analysis are shown in Table 3. There were no significant effects of dietary treatments on the studied variables. Average CP values were 17%, while lambs fed EZE had a slightly higher value (17.8%), whereas the control group registered the lowest (17.2%). Regarding fat content, administration of ENZ in lambs resulted in decreased ($P<0.05$) fat content by 3.9% than other treatments.

Table 3. The effect of adding *Salix babylonica* extracts (SB), exogenous enzymes (EZE) or the mixture of the two (EZE-SB) on parameters of chemical composition of sheep meat

Variables	Treatments				LSD	P value
	Control	EZE	SB	EZE-SB		
CP	17.20	17.81	17.47	17.26	1.491	0.654
Fat	7.15	3.96	5.78	5.60	3.735	0.164
Ash	0.58	1.21	0.86	0.98	0.978	0.345
DM	25.32	25.45	24.80	24.93	2.699	0.871

EZE: exogenous enzymes, SB: *Salix babylonica*, EZE-SB: exogenous enzymes + *Salix babylonica*.

Meat color was significantly different ($P<0.05$) for lightness among treatments (L*), with lambs fed EZE-SB having the highest value when compared to SB, Control and EZE treatments. For the red (a*) and yellow (b*) colours, there were no significant differences among treatments. However, a numerical trend was recorded for the various variables of L*, a* and b*, the lamb fed EZE-SB, which was relatively higher when compared with that of other treatments.

Table 4. The effect of adding *Salix babylonica* extracts (SB), exogenous enzymes (EZE) or a mixture of the two (EZE-SB) on color of sheep meat

Variables†	Treatments				LSD	P
	Control	EZE	SB	EZE-SB		
L	34.84 ^{bc}	34.34 ^{c}	41.42 ^{ab}	44.36 ^{a}	6.708	0.0019
a	12.49	13.58	14.05	15.67	3.481	0.0888
b	4.08	4.80	4.62	5.59	2.0723	0.2072

†L: lightness, a: red colors, b: yellow colors

EZE: exogenous enzymes, SB: *Salix babylonica*, EZE-SB: exogenous enzymes + *Salix babylonica*.

DISCUSSION

Values obtained in this study regarding pH are not in accordance with Hernández *et al*. (2009) and Torrescano *et al*. (2009) who reported initial (6.1 and 6.3) and final (5.8 and 5.5) values, respectively; in Pelibuey sheep. Lawrie (1998) reported that sheep meat has a slightly higher pH than other species. however, results herein are slightly elevated compared to other data in sheep. Accordingly, Ramírez *et al*. (2007) indicated a normal range of 5.8-6.2 for pH values in sheep. In addition, it is recognized that sheep are less susceptible to stress ante-mortem factors which might have contributed to these final pH values. Moderate acidic pH values are known to prevent microbial contamination which favours the preservation of meat. Thus lambs fed EZE-SB with mildly acidic conditions compared to the other treatments are to prove a more favourable condition. It is well known that the final pH as it approaches the protein's isoelectric point (pH 5.0-5.5) will produce gradual decrease in water retention. López (1987), reported that the maximum amount of juice is expelled with higher values of pH in muscles.

Similar results in terms of CP content in Suffolk lamb meat were reported by Pérez *et al*. (2002), with average values being 17% and 18% CP for males and females, respectively.

Results obtained herein regarding meat color are not in agreement to the values reported by Germano *et al*. (2009) who mentioned values for L* of 27.73, which is low compared to our results. Similarly, data from this study is not in accordance to Costa *et al*. (2008) who while performing studies with Nova, Santa Ines and Dorpper x Santa Ines sheep breeds reported values for L* (26.46, 28.99 and 28.00 respectively), for a* (average =12.22) and for b* (average=18.00). The pH has a close relationship with meat color, high pH in meat results in low luminosity (L*). It has been indicated (Hopkins *et al.,* 1995) that there is a significant negative correlation between L* and b* values and muscular pH, which is in accordance to our study, where treatments with high pH yielded low degrees of lightness (L*) such as in groups fed Control and EZE, whereas low pH treatments yielded higher degrees of brightness, as SB and EZE-SB. Accordingly, (EZE-SB). had low pH against higher lightness (L*). In addition, Sañudo

et al. (1998) indicated differences in the variable L* for sheep meat with females and males 39.80 and 41.26, respectively.

CONCLUSION

The combination of SB extract enzymes as additives improved meat pH and color compared to the control or individual additives which resulted in a more favourable color and might contribute to a better conservation profile.

REFERENCES

AOAC. 1990. *Official Methods of Analysis*, 15th ed. Association of Official Analytical Chemists, Arlington, Virginia. p. 13.

Bianchi, G. 2007. *Alternativas Tecnológicas para la Producción de Carne Ovina de Calidad en Sistemas Pastoriles* (Ed. Hemisferio Sur), Montevideo, Uruguay, pp. 190-235.

Brewer, M.S. 1999. Consumer attitudes towards color and marbling of fresh pork. *Food Science*, 64: 171-174.

Costa, R.G., Cartaxo, F.Q., Santos, N.M. and Queiroga, R.C.R. 2008. Goat and sheep meat: fatty acids composition and sensorial characteristics. *Revista Brasileira De Saúde E Producao Animal*, 9: 497-506.

Delfa, R., Lahoz, F. and González, C. 1995. Modelos de clasificación de canales ovinas en la Unión Europea. *Eurocarne*, 37: 37-44.

Gado, H.M., Metwally, H.M., Soliman, H., Basiony, A.Z.L. and El Galil, E.R. 2007. Enzymatic treatments of bagasse by different sources of cellulase enzymes. In: *Proceedings of the 11th Conference on Animal Nutrition,* November 13-18, 2007, Al-Aqsor-Aswan, Egypt, Vol. 10. p. 607.

Gado, H.M. and Salem, A.Z.M. 2008. Influence of exogenous enzymes from anaerobic source on growth performance, digestibility, ruminal fermentation and blood metabolites in lambs fed of orange pulp silage in total mixed ration. In: *Proceedings of the 59th Annual Meeting of the European Association for Animal Production,* August 24-27, Vilnius, Lithuania, p. 228.

Germano, C.R., Malveira, B.A.S., Suely, M.M., Gonzaga, N.S., Ramos, E.Q.C., Araújo, F.J.T. and Selaive, V.A. 2009. Physical and chemical characterization of lamb meat from different genotypes, submitted to diet with different fibre contents. *Small Ruminant Research*, 81: 29-34.

Hernández, C.L., Ramírez, B.J.E., Guerrero, L.M.I., Hernández, M.O., Crosby, G.M.M. and Hernández, C.L.M. 2009. Effects of crossbreeding on carcass and meat quality of Mexican lambs. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 61: 475-483.

Honikel, K.O. 1998. Reference methods for the assessment of physical characteristics of meat. *Meat Science,* 49: 447-457.

Hopkins, D.L., Ferrier, G.R., Channon, H.A. and MacDonald, B.A. 1995. Assessment of lamb meat quality in Sydney and Melbourne. *Proceedings of the New Zealand Society of Animal Production*, 55: 114-116.

Lawrie, R.A. 1998. *Lawrie's Meat Science,* 4th ed, Zaragoza. España, pp. 91-156.

López, M. 1987. *Calidad de la canal y de la carne en los tipos de lechal, ternasco y cordero de la raza Lacha y estudio de su desarrollo*. Thesis Doctoral, Universidad de Zaragoza, Zaragoza, España.

Pérez, P., Maino, M., Tomic, G., Mardones, E. and Pokniak, J. 2002. Carcass characteristics and meat quality of Suffolk Down suckling lambs. *Small Ruminant Research*, 44: 233-244.

Ramirez, B.E., Hernández, C.L., Guerrero, L.I. and Hernández, C.L.M. 2007. Calidad de la carne y análisis sensorial en ovinos de pelo y lana provenientes de engorda intensiva en México. *Vº Congreso de Especialistas en Pequeños Rumiantes y Camélidos Sudamericanos*, Mayo, 2007, Mendoza, Argentina, pp. 1-3.

SAS. 2002. *Users Guide: Statistics* (Release 8.01). SAS Institude Inc. Cary, NC, USA.

Salem, A.Z.M., Salem, M.Z.M., El-Adawy, M.M. and Robinson, P.H. 2006. Nutritive evaluations of some browse tree foliages during the dry season: secondary compounds, feed intake and *in vivo* digestibility in sheep and goats. *Animal Feed Science and Technology,* 127: 251-267.

Salem, A.Z.M., Robinson, P.H., El-Adawy, M.M. and Hassan A.A. 2007. *In vitro* fermentation and microbial protein synthesis of some browse tree leaves with or without addition of polyethylene glycol. *Animal Feed Science and Technology,* 138: 318-330.

Salem, A.Z.M., Robinson, P.H., Lopez, S., Gohar, Y.M., Rojo, R. and Tinoco, J.L. 2010. Sensitivity of sheep intestinal lactic acid bacteria to secondary compounds extracted from Acaciasaligna leaves. *Animal Feed Science Technology*, 61: 85-93.

Sañudo, C., Sierra, I., Olleta, J.L., Martin, L., Campo, M.M., Santolaria, P., Wood, J.D. and Nute, G.R. 1998. Influence of weaning on carcass quality, fatty acid composition and meat quality in intensive lamb production systems. *Animal Science*, 66: 175-187.

Steel, R.G.D. and Torrie, J.H. 1980. *Principles and Procedures of Statistics*. *A Biometrical Approach,* 2nd ed. McGraw Hill Inter. Book Co, Tokyo, Japan.

Torrescano, U.G., Sánchez, E.A., Peñuri, M.F., Velázquez, C.J. and Sierra, R.T. 2009. Características de la canal y calidad de la carne de ovinos pelibuey, engordados en Hermosillo, Sonora. *Biotecnia*, 11: 41-50.

Animal Nutrition and Feed Technology (2013) 13: 381-389

Effects of Various Fibrolytic Enzyme Extracts on Digestibility and Productive Performance of Lambs Fed a Forage-Based Diet[#]

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ABSTRACT

Torres, N., Mendoza, G.D., Bárcena, R., Loera, O., González, S., Aranda, E., Hernández, P.A. and Crosby, M. 2013. Effects of various fibrolytic enzyme extracts on digestibility and productive performance of lambs fed a forage-based diet. Animal Nutrition and Feed Technology, 13: 381-389.

Three fibrolytic enzyme extracts were evaluated for their effects on lamb growth and feed digestibility, using diets containing 60% forage. Twenty Pelibuey lambs (26.14 kg average initial weight) were randomly assigned to a control or to one of three enzyme treatments. Enzymes were added to feed as follows: Control (0 mL kg^{-1} DM), *Trichoderma longibrachiatum* commercial extract (3.5 mL kg^{-1} DM), *Fomes fomentarius* extract (21.1 mL kg^{-1} DM) and *Cellulomonas flavigena* extract (2.47 mL kg^{-1} DM). There were no effects of fibrolytic enzyme extracts on the final weight of lambs or on dry matter intake, but daily weight gain and feed efficiency were negatively affected ($P<0.05$) by *Cellulomonas flavigena* extract. The addition of enzyme extracts increased ($P<0.05$) the percentage of butyrate in rumen fluid. The DM digestibility was negatively affected by *C. flavigena* extract. There were no beneficial effects of fibrolytic enzyme extracts on feed digestibility or productive performance of lambs.

Key words: *Cellulomonas flavigena*, Digestibility, Fibrolytic enzymes, *Fomes fomentarius*, *Trichoderma longibrachiatum*.

INTRODUCTION

One of the applications of biotechnology to ruminant nutrition has been the use of exogenous fibrolytic enzymes to improve the digestibility of structural carbohydrates in high quality forages (Colombatto *et al.*, 2003; Dean *et al.,* 2008). These enzymes are

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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produced commercially from *Aspergillus oryzae*, *Aspergillus niger*, *Trichoderma reesei* and *Trichoderma viride* (Nadeau *et al.*, 2000; McAllister *et al.,* 2001). However, some studies have shown inconsistent results when these products are added to low quality forages (Avellaneda *et al.*, 2009; Carreón *et al.*, 2010; Gallardo *et al.*, 2010) with high NDF content and low ruminal digestibility (Tan *et al.,* 1996; Alvarez and Combellas, 2005). Therefore, there is a need to identify enzyme extracts capable of improving digestibility of this type of lignocellulosic substrate. Some previously evaluated enzymes include those originating from the white rot fungi *Fomes fomentarius* (Villegas-Castañeda *et al.,* 2010) and from the bacterium *Cellulomonas flavigena* (Hernández *et al.,* 2011). These studies suggest that the *in vitro* or *in situ* ruminal digestibility of straw is improved when extracts from these organisms are included in the diet (Márquez-Araque *et al.*, 2007; Hernández, 2009; Villegas-Castañeda *et al.*, 2010). However, studies with animals are scarce, and thus the objective of this experiment was to evaluate whether fibrolytic enzyme extracts from *C. flavigena* and *Fomes fomentarius* and a commercial extract from *Trichoderma longibrachiatum* could improve intake, digestibility or weight gain of lambs fed a ration with 60% forage.

MATERIALS AND METHODS

Animals and feeding

The study was conducted at the Postgraduate College, Montecillo Campus, Mexico, under the supervision of the Academic Committee of the Department of Animal Science in accordance with the rules established by the Animal Protection Law of the State of Mexico. Twenty Pelibuey crossbred lambs (26.14±1.73 kg initial weight; mean±SD) were randomly distributed into four treatment groups using individual metabolic cages. All animals were dewormed with Ivermectin (commercial name and laboratory) prior to the trial, and vitamins (A, D and E; commercial name and laboratory) were offered during the 10 d adaptation period. These supplements were provided in two meals (0900 and 1700 h). The basic diet was formulated according to NRC recommendations (1985), containing 60% forage (30% alfalfa hay and 30% corn stover) and 40% concentrate (15% corn, 10% sorghum, 6% soybean meal, 7% molasses, 1% urea and 1% mineral mix). This ration was analysed for dry matter (DM, 918.8 g kg^{-1}) and crude protein (PC, 156.4 g kg^{-1}) following the procedures of the AOAC (2005) and for neutral detergent fiber (FDN, 420.4 g kg^{-1}) and acid detergent fiber (FDA, 247.4 g kg^{-1}) according to the procedures described by Van Soest *et al.* (1991).

Enzymes

The enzymes used were from *T. longibrachiatum* (Promote NET, Cargill Corp., Minneapolis, MN, USA), *Fomes fomentarius* and *C. flavigena*. The fungal extract was obtained from solid fermentation growth on corn stover (Villegas-Castañeda *et al.*, 2010), and *C. flavigena* was obtained by liquid fermentation of sugarcane bagasse

(Vega-Estrada *et al.*, 2002). The protein concentration (Bradford, 1976) and cellulolytic and xylanolytic activity in the extracts (Loera and Cordova, 2003) were determined by expressing the enzymatic activity (IU) per mL of extract, with one IU defined as 1 mol of xylose or glucose released per mL extract per minute (Table 1). With this information, the enzyme dose was selected (mL of extract kg^{-1} DM of feed) to produce equivalent xylanolytic activity.

Table 1. Enzymatic activity and protein content of fibrolytic enzyme extracts

Items	Source of enzymes			SEM†
	Trichoderma longibrachiatum	*Fomes fomentarius*	*Cellulomonas flavigena*	
Enzymatic activities, IU				
Cellulases‡	3.47	2.85	2.66	1.19
Xylanases§	13.57	2.25	19.19	1.04
Laccases¶	-	0.021	-	0.003
Protein, mg mL^{-1}	0.49	0.31	0.46	0.01

†Standard error of the mean; ‡μmol of glucose released mL min^{-1}; §μmol of xylose released mL min^{-1}; ¶μmol of ABTS oxidized mL min^{-1}

Treatment

The treatments comprised doses equivalent to 47.50 IU of extract xylanolytic activity per kg DM of feed as follows: a) control group (0 mL kg^{-1} DM); b) *T. longibrachiatum* extract (3.5 mL kg^{-1} DM); c) *F. fomentarius* extract (21.1 mL kg^{-1} DM); and d) *C. flavigena* extract (2.47 mL kg^{-1} DM). The enzymes were diluted in 300 mL water and sprayed on the forage daily, before the forage was mixed with other feeds and offered to the lambs.

Productive performance

The trial lasted 42 days; lambs were weighed every 14 days after 12 h of fasting to estimate average daily gain (ADG, g d^{-1}) and feed conversion. Feed consumption (kg DM d^{-1}) was recorded on daily basis. In the middle of the trial, total faeces were collected from each animal over 24 h periods for 5 consecutive days to determine DM digestibility using the internal marker, acid insoluble ash concentration (Keulen and Young, 1977). NDF and ADF digestibility were also determined (Van Soest *et al.,* 1991).

Ruminal variables

A sample of 50 mL of rumen fluid was collected from each lamb using an esophageal probe during the last day of testing. The pH was measured immediately, and the rest of the rumen fluid was preserved by acidification with 2 mL of 25% metaphosphoric acid. The samples were kept frozen until analysis of VFA by gas chromatography (Erwin *et al.*, 1961) and NH_3-N by spectrophotometry, as previously described by McCullough (1967).

Statistical analysis

The data were analysed as a completely randomized design with four treatments and five replications. The GLM procedure of SAS (2002) was used, and the means were compared with the Tukey test.

RESULTS AND DISCUSSION

Productive variables and digestibility

No enzyme effects ($P > 0.10$) were observed on the final lamb weights or on dry matter intake. The daily weight gain and feed conversion were higher ($P < 0.05$) in the control and *F. fomentarius* treatments (Table 2) compared to other enzyme treatments. The digestibility of DM, NDF and ADF decreased ($P < 0.01$) in lambs receiving *C. flavigena* extract compared to the control and other enzyme treatments (Table 3).

Table 2. Effect of fibrolytic enzymes on the productive performance of finishing lambs

Items	Enzymes				SEM†	P value
	Control	*Trichoderma longibrachiatum*	*Fomes fomentarius*	*Cellulomonas flavigena*		
Initial BW (kg)	25.68	26.26	26.16	26.46	0.87	0.93
Final BW (kg)	34.24	32.94	34.74	32.62	1.14	0.52
Intake (g DM d^{-1})	1082	1030	1124	1116	53	0.60
ADG (g d^{-1})	220^{b}	158^{c}	206^{bc}	160^{c}	17	0.04
Feed conversion	5.17^{b}	7.78^{bc}	5.76^{b}	7.53^{c}	0.57	0.01

†Standard error of the mean

^{bc}Within a row, means denoted by different superscript letters are significantly different ($P < 0.05$)

Table 3. Effect of fibrolytic enzymes on the digestibility of rations fed to finishing lambs

Items	Enzymes				SEM†	P value
	Control	*Trichoderma longibrachiatum*	*Fomes fomentarius*	*Cellulomonas flavigena*		
DM (g kg^{-1})	770.1^{b}	744.9^{b}	724.4^{b}	605.1^{c}	18.4	0.0001
NDF (g kg^{-1})	703.6^{b}	675.0^{b}	644.6^{b}	527.8^{c}	21.6	0.0002
ADF (g kg^{-1})	598.7^{b}	598.8^{b}	586.7^{b}	405.4^{c}	26.67	0.0002

†Standard error of the mean

^{bc}Within a row, means denoted by different superscript letters are significantly different ($P < 0.05$)

Feed intake and feed efficiency values were similar to those reported by several authors who used exogenous fibrolytic enzymes without observing changes in DM intake in ruminants (Krause *et al.*, 1998, Pinos *et al.*, 2002; Wang *et al.*, 2004; Pinos *et al.,* 2008). In experiments on sheep using diets with a high proportion of forage and doses of 12 g d^{-1} of *Aspergillus niger* and *T. longibrachiatum* enzymes, no effects on DM digestibility and feed intake were detected (Giraldo *et al.,* 2008). However, in

other studies on steers with higher doses of *T. longibrachiatum* enzymes (60 g d^{-1}), weight gain and feed efficiency were improved (Balci *et al.,* 2007), indicating that the dose may be a factor in the response.

The use of *Trichoderma* enzymes in both *in vitro* and *in vivo* fermentation showed increased microbial activity depending on the substrate and the dose used (Wallace *et al.,* 2001; Martins *et al.,* 2006; Giraldo *et al*., 2008; Gomez-Vazquez *et al.,* 2011). When the enzymes acted on the cell wall, forage digestibility increased (Castro *et al*., 2004; Gomez-Vazquez *et al.,* 2011). The effect of the addition of *F. fomentarius* enzymes on ruminant rations was evaluated *in vivo*, and *in vitro* incubations have also shown beneficial effects of exogenous enzymes of this fungus (Levin and Forchiassin, 1997; Márquez-Araque *et al*., 2007). Similar results have been observed with *C. flavigena* extract under *in situ* conditions, with increased digestibility and increased agricultural wastes reported when extracts of this bacterium were added to alfalfa hay as substrates (Hernandez, 2009). The results of the current study showed no beneficial effects *in vivo* for either extract.

Ruminal fermentation

Extracts from *C. flavigena* and *T. longibrachiatum* increased ($P<0.05$) the percentage of butyrate relative to the control, while the butyrate percentage of the *F. fomentarius* extract treatment was intermediate (Table 4). Acetate, propionate, ammonia-N and ruminal pH were not affected by exogenous enzymes.

Table 4. Effect of fibrolytic enzymes on ruminal fermentation variables in finishing lambs

Items	Enzymes				SEM†	P value
	Control	*Trichoderma longibrachiatum*	*Fomes fomentarius*	*Cellulomonas flavigena*		
pH	6.74	6.59	6.83	6.86	0.11	0.32
NH_3-N, mg dL^{-1}	9.48	10.22	6.96	9.98	1.82	0.58
Volatile fatty acids, (mol 100 mol^{-1})						
Acetic	62.44	60.24	63.45	60.84	0.96	0.11
Propionic	26.86	25.27	23.16	25.11	1.05	0.14
Butyric	10.70^{c}	14.49^{b}	13.38^{bc}	14.05^{b}	0.91	0.04
Total VFA, mM	72.11	93.32	79.09	91.94	7.80	0.20

†Standard error of the mean

[bc]Within a row, means denoted by different superscript letters are significantly different ($P<0.05$)

Various studies report findings similar to those of this experiment regarding the lack of response of pH, NH_3-N and AGV (Hristov *et al*., 2000; Pinos *et al*., 2002; Beauchemin *et al*., 2003) to *A. niger* and *T. longibrachiatum* enzymes. However, Giraldo *et al*. (2007) reported higher production of acetic and butyric acids *in vitro* when using *T. longibrachiatum* enzymes with 70% grass hay and 30% concentrate as substrates, with no changes in pH or propionic acid production. In addition, Villegas-

Castañeda *et al*. (2010) used *F. fomentarius* enzymes for *in vitro* gas production, using sorghum straw as a substrate, and reported no changes in total VFA following the addition of the enzyme.

The increased butyric acid observed in this experiment promoted the release of simple carbohydrates such as glucose, possibly as a result of the presence of the *T. longibrachiatum* and *C. flavigena* fibrolytic enzymes. These enzymes promote the populations of protozoa that produce butyrate (Luther *et al.*, 1966; Williams *et al.*, 1986), in particular, *Dasytricha ruminantium* (Yarlett *et al*., 1985). Considering that an increase in fiber digestibility, but not the associated production of butyrate, increased the activity of the bacteria Butyrivibrio fibrisolvens (Diez-Gonzalez et al., 1999; Marounek and Duskova, 1999), this indicates another major micro-organism that can increase butyrate in the rumen.

The decrease in digestibility with the addition of C. flavigena extract could be associated with the release of glucose, which inhibits the activity of cellulolytic enzymes of some rumen micro-organisms, such as fungi (Barichievich and Calza, 1990). It was reported that C. flavigena could produce the enzymes endo-1,4-β-xylanase, β-glucosidase and glucoronoarabinoxylan (Martinez-Trujillo *et al.*, 2003; Barrera-Islas *et al.,* 2007; Mejia-Castillo *et al.,* 2008; Perez-Avalos *et al.,* 2008).

CONCLUSION

The addition of enzymes obtained from *F. fomentarius* and *C. flavigena* to forage-based diets of lambs had no beneficial effects on the productive performance of sheep, and feed conversion was adversely affected by an increase in butyric acid. The extract of *C. flavigena* negatively affected the DM digestibility *in vivo*.

REFERENCES

Álvarez, R. and Combellas, J. 2005. Evaluation of poultry litter on sorghum straw intake and dry matter disappearance using dry cows. *Revista Brasileira de Zootecnia*, 34: 584-588.

AOAC. 2005. *Official Methods of Analysis,* 18th ed. Association of Official Analytical Chemists, Washington, DC, USA.

Avellaneda, J.H., Pinos-Rodríguez, J.M., González-Muñoz, S.S., Bárcena R., Hernández, A., Cobos, M., Hernández, D., Montañez and D.O. 2009. Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestion of guineas grass hay. *Animal Feed Science and Technology,* 149: 70-77.

Balci, F., Dikmen, S., Gencoglu, H., Orman, A. Turkmen, I.I. and Biricik, H. 2007. The effect of fibrolytic exogenous enzymes on fattening performance of steers. *Bulgarian Journal of Veterinary Medicine*, 10: 113-118.

Barichievich, E.M. and Calza, R.E. 1990. Supernatant protein and cellulase activities of the anaerobic ruminal fungus *Neocallimastix frontalis* EB188. *Applied and Environmental Microbiology*, 56: 43-48.

Barrera-Islas, G.A., Ramos-Valdivia, A.C., Salgado, L.M. and Ponce-Noyola, T. 2007. Characterisation of a beta-glucosidase produced by a high-specific growth-rate mutant of *Cellulomonas flavigena*. *Current Microbiology*, 54: 266-270.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilisation by ruminants. *Journal of Animal Science,* 81: E37-47.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry,*72: 248-252.

Carreón, L., Pinos-Rodríguez, J., Bárcena R., González, S. and Mendoza G. 2010. Influence of fibrolytic enzymes on ruminal disappearance and fermentation in steers fed diets with short and long particle length of forage. *Italian Journal of Animal Science,* 9: e17.

Castro, A.L. De Aguiar Paiva, P.A., Souza Dias, E. and Dos Santos, J. 2004. Nutritional value and degradability alterations of cotton textile mill waste after biological treatment with *Pleurotus sajorcaju*. *Ciência e Agrotecnologia Lavras*, 28: 608-613.

Colombatto, D., Morgavi, D.P., Furtado, A.F. and Beauchemin, K.A. 2003. Screening of exogenous enzymes for ruminant diet: relationship between biochemical characteristics and *in vitro* ruminal degradation. *Journal of Animal Science,* 81: 2628-2638.

Dean, D.B., Adesogan, A.T., Krueger, N.A. and Littell, R.C. 2008. Effects of treatment with ammonia or fibrolytic enzymes on chemical composition and ruminal degradability of hays produced from tropical grasses. *Animal Feed Science and Technology,* 145: 68-83.

Diez-Gonzalez, F., Bond, D.R., Jennings E. and Russell J.B. 1999. Alternative schemes of butyrate production in *Butyrivibrio fibrisolvens* and their relationship to acetate utilisation lactate production, and phylogeny. *Archives of Microbiology*, 171: 324-330.

Erwin, E.S., Marco, G.T., Emery, E.M., 1961. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. *Journal of Dairy Science,* 44: 1768-1771.

Gallardo, I., Bárcena, R., Pinos-Rodríguez, J.M., Cobos, M., Carreón, L. and Ortega, M.E. 2010. Influence of exogenous fibrolytic enzymes on *in vitro* and *in sacco* degradation of forages for ruminants. *Italian Journal of Animal Science,* 9: e8.

Giraldo L.A., Tejido M.L., Ranilla M.J., Ramos S. and Carro M.D. 2008. Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. *Journal of Animal Science,* 86: 1617-1623.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2007. Effects of exogenous cellulase supplementation on microbial growth and ruminal fermentation of a high-forage diet in Rusitec fermenters. *Journal of Animal Science,* 85: 1962-1970.

Hernández, G.P.A. 2009. *Caracterización del Extracto Enzimático Xilanolítico Exógeno de Cellulomonas flavigena en Condiciones Ruminales*. PhD Thesis, Colegio de Postgraduados, México.

Hernández, P.A., Bárcena J.R., Mendoza G.D., Montes C.S., González S.S. and Rojo R. 2011. Xylanase activity from *Celluomonas flavigena* extracts as affected by temperature and its degradation under *in vitro* ruminal conditions. *African Journal of Microbiology Research*, 5: 961-964.

Hristov, A.N., McAllister, T.A. and Cheng, K.J. 2000. Intra-ruminal supplementation with increasing levels of exogenous polysaccharidase-degrading enzymes: Effects on nutrient digestion in cattle fed a barley grain diet. *Journal of Animal Science,* 78: 477-487.

Gómez-Vázquez, Mendoza, G.D., Aranda, E., Pérez, J., Hernández, A. and Pinos-Rodríguez, J.M. 2011. Influence of fibrolytic enzymes on growth performance and digestion in steers grazing stargrass and supplemented with fermented sugarcane. *Journal of Applied Animal Research,* 39: 77-79.

Keulen, J.V. and Young. B.A. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *Journal of Animal Science,* 44: 282-287.

Krause, M., Beauchemin, K.A., Rode, L.M., Farr, B.I. and Norgaard, P. 1998. Fibrolytic enzyme treatment of barley grain and source of forage in high-grain diets fed to growing cattle. *Journal of Animal Science,* 76: 2912-2920.

Levin, L. and Forchiassin, F. 1997. Efecto de las condiciones de cultivo sobre la producción de celulasas por *Trametes trogii*. *Revista Argentina de Microbiología*, 29: 16-23.

Lewis, G.E., Hunt, C.W., Sanchez, W.K., Treacher, R., Pritchard, G.T. and Feng, P. 1996. Effect of direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. *Journal of Animal Science,* 74: 3020-3028.

Loera, O, and Córdova, J. 2003. Improvement of xylanase production by a parasexual Cross between *Aspergillus niger* strains. *Brazilian Archives of Biology and Technology*, 46: 177-181.

Luther, R., Treakle, A. and Burroughs. W. 1966. Influence of rumen protozoa on volatile acid production and ration digestibility in lambs. *Journal of Animal Science* 25: 1116-1122.

Márquez-Araque, A.T., Mendoza-Martínez, G.D., González-Muñoz, S.S., Búntinx-Dios, S.E. and Loera-Corral, O. 2007. Actividad fibrolítica de enzimas producidas por *Trametes* sp. EUM1, *Pleurotus ostreatus* IE8 y *Aspergillus niger* AD96.4 en fermentación sólida. *Interciencia*, 32: 780-785.

Martínez-Trujillo, A., Pérez-Avalos, O. and Ponce-Noyola, T. 2003. Enzymatic properties of a purified xylanase from mutant PN-120 of *Cellulomonas flavigena*. *Enzyme Microbial and Technology*, 32: 401-406.

Martins, A.S., Vieira, P.F., Berchielli, T.T., Prado I.N. and Moletta, J.L. 2006. Intake and apparent total tract digestibility in cattle supplemented with fibrolytic enzymes. *Revista Brasileira de Zootecnia*, 35: 2118-2124.

Marounek, M. and Duškov, D. 1999. Metabolism of pectin in rumen bacteria *Butyrivibrio fibrisolvens* and *Prevotella ruminicola*. *Letters of Applied Microbiology*, 29: 429-433.

McAllister, T.A., Hristov, A.N., Beauchemin, K.A., Rode, L.M. and Cheng, K.Jr. 2001. Enzymes in ruminants diets. In: *Enzymes in Farm Animal Nutrition* (Eds. M. Bedford and G.G. Partridge). CABI Publishing, Oxon, UK.

McCullough, H., 1967. The determination of ammonia in whole blood by direct colourimetric method. *Clinical Chemistry,* 17: 297-304.

Mejia-Castillo, T., Hidalgo-Lara, M.E., Brieba, L.G. and Ortega-Lopez, J. 2008. Purification, characterisation and modular organisation of a cellulose-binding protein, CBP105, a processive beta-1,4-endoglucanase from *Cellulomonas flavigena*. *Biotechnology Letters*, 30: 681-687.

Nadeau, E.M., Rusell, J.R. and Buxton, D.R. 2000. Intake, digestibility and composition of orchard grass and alfalfa silage treated with celullase, inoculants, and formic acid fed to lambs. *Journal of Animal Science,* 78: 2980-2989.

NRC. 1985. *Nutriment Requirements of Sheep*, 6th ed. National Research Council, National Academic Science, Washington, DC, USA.

Pérez-Avalos, O., Sánchez-Herrera, L.M., Salgado, L.M. and Ponce-Noyola, T. 2008. A bifunctional endoglucanase/endoxylanase from *Cellulomonas flavigena* with potential use in industrial processes at different pH. *Current Microbiology*, 57: 39-44.

Pinos-Rodríguez, J.M., González, S.S., Mendoza, G.D., Bárcena, R., Cobos, M.A., Hernández, A. and Ortega, M.E. 2002. Effects of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. *Journal of Animal Science,* 80: 3016-3020.

Pinos-Rodríguez, J. M., Moreno, R., González, S.S., Robinson, P.H., Mendoza, G. and Álvarez, G. 2008. Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestibility of total mixed rations fed to lambs. *Animal Feed Science and Technology,* 142: 210-219.

SAS. 2002. *SAS User's Guide: Statistics*. Version 8.02. Cary, NC, SAS Inst.

Tan, Z.L., Chen, H.P. and Xing. T.X. 1996. Comparative study on fibre characteristics of rice and wheat straw. *Asian-Australasian Journal of Animal Science,* 9: 51-56.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fibre, neutral detergent fibre, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3592.

Vega-Estrada J., Flores-Contreras, L.B., Santiago, A., Magaña-Plaza, I. and Montes-Horcasitas, C. 2002. Draw-fill batch culture mode for production of xylanases by *Cellulomonas flavigena* on sugar cane bagasse. *Applied Microbiology and Biotechnology*, 58: 435-438.

Villegas-Castañeda, M., Meneses-Mayo, M., Miranda-Romero, L.A. and Loera-Corral, O. 2010. Producción de gas *in vitro* y desaparición de la matera seca del cultivo sólido con hongos ligninolíticos. *Agrociencia*, 44: 917-929.

Wallace, J.R., Wallace, A.J.S., McKain, N., Nsereko, L.V. and Hartnell, F.G. 2001. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal micro-organisms *in vitro*. *Journal of Animal Science,* 79: 1905-1916.

Wang, Y., Spratling, B.M., ZoBell, D.R., Wiedmeier, R.D. and McAllister, T.A. 2004. Effect of alkali pre-treatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. *Journal of Animal Science,* 82: 198-208.

Williams, A.G., Ellis, A.B. and Coleman, G.S. 1986. Subcellular distribution of polysaccharide depolymerase and glycoside hydrolase enzymes in rumen ciliate protozoa. *Current Microbiology*, 13: 139-147.

Yarlett, N., Lloyd, J.D. and Williams, A.G. 1985. Butyrate formation from glucose by the rumen protozoon *Dasytricha ruminantium*. *Biochemistry Journal*, 228: 187-192.

Animal Nutrition and Feed Technology (2013) 13: 391-398

Effects of Exogenous Glucoamylase from *Aspergillus niger* and Grain Level on Performance of the Lambs[#]

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ABSTRACT

Mendoza, G.D., Mota, N., Plata, F.X., Martinez, J.A. and Hernández, P.A. 2013. Effects of exogenous glucoamylase from Aspergillus niger and grain level on performance of the lambs. Animal Nutrition and Feed Technology, 13: 391-398.

The objective of this study was to evaluate the effects of the addition of glucoamylase and the reduction of the level of sorghum grain in a finishing diet on lamb performance (45 days) and on diet digestibility. The treatments evaluated were the following: a diet composed of 64% corn and sorghum grain mixture (64CSMG), a diet composed of 45% corn and sorghum grain mixture (45CSMG) and a diet composed of 45% corn and sorghum grain mixture plus added glucoamylase (1.5 ml of the enzyme protein/kg DM of grain; 45CSMG+E). *In vivo* DM digestibility showed differences ($P<0.05$) between treatments; the highest values were for the 64% grain diet (748 g/kg) and the 45% grain with enzyme diet (694 g/kg), and the lowest value was for the 45% grain without enzyme diet (740 g/kg). There were no differences ($P> 0.05$) in dry matter intake, average daily gain (ADG) and feed conversion even when the digestibility of the 45% grain ration with glucoamylase was improved.

Key words: *Aspergillus niger*, Exogenous enzymes, Glucoamylase, Grain, Lambs' performance.

INTRODUCTION

Certain processes have been utilized to improve starch digestion, such as flaking or rolling, but those are expensive; thus, there are strategies to improve the ruminal digestibility of starch that include the addition of exogenous amylolytic enzymes (Rojo *et al.*, 2005; Weiss *et al.*, 2011).

Exogenous amylolytic enzymes have been evaluated in high-grain diets (Rojo *et al.*, 2005; Gutierrez *et al.*, 2005); however, most of the studies have been conducted with fibrolytic enzymes (Colombatto *et al.*, 2003a, b; Salem *et al.*, 2011). Initially, the *in vitro* studies helped identify enzymes resistant to degradation by ruminal proteases and their effects on the digestibility of nutrients and substrates (Colombatto *et al.*, 2003a, b). Evaluations have been made of the effects of exogenous fibrolytic enzymes

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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on nutrient digestibility *in vitro (*Eun *et al.*, 2006a, b; Eun and Beauchemin, 2007), on the milk production response and on goat and sheep production (Schingoethe *et al.*, 1999; Pinos *et al.*, 2005; Salem *et al.*, 2011); a few experiments with amyolytic enzymes in high grain diets have also been reported (Rojo *et al.*, 2005; Mota *et al.*, 2011).

A potential benefit of amyolytic enzymes is that the amount of grain in the ration may be reduced without impairing the productive response of animals. This strategy has been used in dairy cattle with good results with fibrolytic (Schingoethe *et al.*, 1999) and amylolytic enzymes (Gencoglu *et al.*, 2010), but studies related to high-grain diets are scarce (Mota *et al.*, 2011). Thus, the objective of this study was to evaluate the potential use of a glucoamylase enzyme to improve diet digestibility and lamb performance when the level of grain in the diet is reduced.

MATERIALS AND METHODS

Treatments

The dietary treatments consisted of the following: 1) a control diet composed of 64% corn and sorghum grain mixture (64CSMG); 2) a diet composed of 45% corn and sorghum grain mixture (45CSMG); and 3) a diet composed of 45% corn and sorghum grain mixture with an added enzyme (1.5 ml of the enzyme protein/kg DM of grain; 45CSMG+E; Table 1). In the experimental diets, dry matter (DM), organic matter (OM), crude protein (CP; NX6.25), ether extract (EE; AOAC, 1990), neutral detergent fiber (NDF; Van Soest *et al.*, 1991) and starch (Herrera and Huber, 1989) levels were determined. The enzyme tested was a glucoamylase from *Aspergillus niger* (Glucozime L-400® produced by ENMEX, Mexico). The Amylolytic activity of this enzyme has been reported in other studies (Rojo *et al.*, 2005) when it was diluted in water and sprinkled onto ground sorghum grain that was then mixed with other diet ingredients (Mota *et al.*, 2011). Because the enzyme is stable in low humidity conditions (Gutierrez *et al.*, 2005), the diet was prepared weekly.

In vitro and in situ experiment

In vitro DM digestibility of the dietary treatments was measured using the Tilley and Terry (1963) technique. The incubation procedure was repeated 3 times in different weeks (Pinos *et al.*, 2002) and samples were incubated for 6, 12 and 24 h. The ruminal fluid was obtained in different weeks from a Holstein-Friesian ruminally cannulated cow (420 kg BW) that was fed alfalfa hay *ad libitum* and 1.9 kg (DM) of concentrate (Unión Tepexpán®; 14.5% CP, Starch 51.5%, ME 3.5 Mcal/kg) per day. Dietary treatments were also evaluated by *in situ* digestibility by incubating samples for 0, 6, 12 and 24 h following the procedure modified by Vanzant *et al.* (1998) and using the same ruminally cannulated cow. Incubations were replicated 3 times to use incubation procedures as the blocking criteria (Pinos *et al.*, 2002).

Table 1. Composition of experimental diets

	Treatment		
	64CSMG	45CSMG	45CSMG+E
Ingredient composition			
Ground corn, %	40	30	30
Ground sorghum, %	24	15	15
Soybean meal, %	5.5	6	6
Alfalfa hay, %	4	12	12
Corn gluten meal,%	3.5	3	3
Molasses, %	10	10	10
Corn stover, %	11	22	22
Minerals[†], %	1.5	1.5	1.5
Buffer[‡], %	0.5	0.5	0.5
Chemical composition			
Dry matter, g/kg	838	837	839
Organic matter, g/kg	959	964	964
Crude protein, g/kg	122	126	123
Ether extract, g/kg	25.5	20.5	20.9
Neutral detergent fiber, g/kg	223	277	306
Starch, g/kg	468	371	372

[†]Sales Chapingo; Ca: 23.7 g., P: 18.3 g., Mg: 0.8 g., NaCI: 3.6 g., Fe: 168 mg., Zn: 378 mg., Cu: 100 mg., I: 8 mg., Co: 15 mg., Mo: 2 mg., Mn: 241 mg., Se: max. 0.06 ppm., vitamin A: 30.000 IU, vitamin D_3: 5.000 IU, vitamin E: 10 mg

[‡]Acid buf®: Ash 95%, Ca 30%, Mg 5%, S 0.5%, Na 1.2%, P 575 ppm, K 650 ppm, B 9.5 ppm, F 190 ppm, Fe 1825 ppm, Co 4 ppm, Cu 16 ppm, Zn 25 ppm, Mo 2.25 ppm, Se 1 ppm, I 160 ppm, vitamin A, vitamin B1, vitamin D, vitamin E, calcium carbonate, cobalt carbonate, sodium chloride, EDDI, calcium monophosphate, magnesium oxide, manganese oxide, zinc oxide, sodium selenite, copper sulfate, iron sulfate, monensin and antioxidant.

Fattening assay and in vivo digestibility

Twenty-one male Suffolk crossbred lambs with initial body weights (BW) of 22.7±2.5 kg were housed in individual metabolic cages. At the beginning of the experiment, the lambs were weighed, dewormed with Ivermectine 1% (1 ml/50 kg BW) and dosed with vitamins A, D and E. The fattening assay lasted 45 days with 12 days for adaptation to the grain diets. Lambs were assigned randomly to each treatment and fed with the corresponding ration twice a day allowing for ±100 g (DM) of orts. The intake and the orts were recorded daily. The BW was recorded every 15 days to estimate average daily gain (ADG). Feed conversion was calculated as DM intake divided by ADG. Apparent *in-vivo* DM digestibility was estimated during the last 5 days of the experiment by collecting one faecal grab sample per animal on 5 consecutive days; these were dried at 55°C for 72 h and ground in a mill with a 1 mm sieve. Feed and faeces were used to determine acid insoluble ash level as an internal marker to estimate digestibility (Keulen and Young, 1977).

Acidosis clinical episodes reduced the pH of the ruminal fluid and this reduction became greater as the problem was repeated, some studies have shown that the ruminal pH after four clinical acidosis episodes is 0.5 (6.05 vs 5.59) lower (Gonzalez *et al.*,

2012) in dairy cattle. For this reason, at the end of the study on day 45, a sample of ruminal fluid was collected using an esophageal tube before feeding (0800 h), and the Ruminal pH was measured immediately with a portable pH meter (Hanna®).

Statistical analysis

Results for *in vitro* and *in situ* digestibility were analysed as a randomized completely block design using the block by treatment interaction as an error term (Pinos *et al.*, 2005). For the fattening trial, results were analysed as a completely randomized design. If variables were measured more than once (in-vitro and *in situ* digestibility), the statistical analysis included the repeated measurements procedure (Herrera and Barreras, 2005). The ADG was analysed with the initial BW as a covariate (Rojo *et al.*, 2005). The means were compared using Tukey's test (Herrera and Barreras, 2005).

RESULTS

In vitro and in situ experiments

In vitro DM digestibility was higher ($P<0.05$) at 24 hours for the 65CSMG diet, intermediate in the ration containing 45CSMG+E and lower in the ration of 45CSMG (Table 2). For the *in situ* results, the diet with the highest starch content (65CSMG) had similar degradability as the 45CSMG+E, with the lowest degradability ($P=0.01$) for the ration with the lowest level of grain without the enzyme (Table 2).

Table 2. Effects of glucoamylase[†] enzyme and reduced grain diet on *in vitro* and *in situ* dry matter digestibility at different incubation times

	Treatment			SEM[‡]	P value[§]
	64CSMG	45CSMG	45CSMG+E		
In vitro DM digestibility, %					
6 h	31.34	24.42	24.85	3.19	0.240
12 h	34.84	44.54	42.80	4.95	0.210
24 h	64.09 [a]	60.33[c]	62.25 [b]	0.17	0.001
In situ DM digestibility, %					
6 h	52.64	46.81	46.61	2.15	0.10
12 h	56.42	56.95	57.07	2.55	0.97
24 h	74.79[a]	64.07 [b]	69.32 [ab]	1.90	0.017

[abc]Means within rows with different superscript differ ($P<0.05$)

[†]Glucoamylase: 1.50 ml enzyme protein/kg DM grain

[‡]SEM: Standard error of the mean

[§]P: Probability of error type I

Fattening assay and in vivo digestibility

There were no differences in lamb performance among treatments; however, the treatment with the enzyme showed a trend ($P=0.08$) to be lower (Table 3). The intake showed a trend ($P=0.06$) to be reduced in the 64CSMG diet; however, there were no

differences in feed conversion. There were differences in DM digestibility (P=0.04); the 64CSMG intermediate and the 45CSMG+E diets showed the highest levels of digestibility and the 45CSMG diet showed the lowest. Ruminal pH was not affected by the treatments (Table 3).

Table 3. Effect of the addition of glucoamylase[†] enzyme and reduced grain diet on the performance of finishing sheep and ruminal pH

	Treatment			SEM[‡]	P value[§]
	64CSMG	45CSMG	45CSMG+E		
Initial body weight, kg	23.55	24.10	24.13	0.93	0.01
Final body weight, kg	34.24	34.55	33.43	0.87	0.01
ADG, kg/d	0.250	0.250	0.220	0.49	0.08
DM intake, kg/d	1.16	1.27	1.19	0.59	0.06
Starch intake, kg/d	0.542[a]	0.442[b]	0.471[b]	0.03	0.14
NDF intake, kg/d	0.258[b]	0.364[a]	0.351[a]	0.02	0.01
Feed conversion, kg/kg	4.66	5.29	5.37	0.30	0.12
Ruminal pH	6.47	6.38	6.34	0.10	0.70
In vivo DM digestibility g/kg	747[a]	726[b]	756[a]	0.79	0.04

[†]Glucoamlyase: 1.50 ml enzyme protein/kg DM grain

[‡]SEM: Standard error of the mean

[§]P: Probability of error type I

[ab]Means within rows with different superscripts differ (P<0.05)

DISCUSSION

As in other *in vitro* experiments in which amylolytic enzymes have been used, *in situ* digestibility improved almost five points (Rojo *et al.*, 2007), but *in vitro* digestibility was increased only two units after 24 h of incubation in this experiment. In other studies, the magnitude of the digestion response was more pronounced because they used a thermostable amylase from *Bacillus licheniformis* with higher activity compared to the glucoamylase from *Aspergillus niger* (Rojo *et al.*, 2005). The *in vitro* digestibility using different doses (0.6 to 3.0 g protein/kg grain) of an amylase from *Bacillus licheinformis* (Crosby *et al.*, 2006) improved DM digestibility (P<0.05). In contrast, Weiss *et al.* (2011) found no effects when a glucoamylase was added to the feed of lactating cows, which indicated that the size and physical form of the grain may affect the response to the exogenous enzymes. However, Dhital *et al.* (2010) showed no relationship between the size of the starch granule and enzymatic digestibility. There are studies that indicate that other factors may affect digestibility and the response to enzymes, such as the amylase concentration, the degree of starch crystallinity, the temperature of exposure and the enzyme dose (Htoon *et al.*, 2009).

The results of the *in vivo* and *in situ* digestibility study confirm that glucoamylase can be used to increase starch digestion (Rojo *et al.*, 2007). The stability of enzymes in the rumen must be characterized in exogenous enzymes (Klingerman *et al.*, 2009) and this may explain why certain enzymes show more response than others. The ruminal pH is another factor that may positively or negatively affect the activity of exogenous

enzymes in the rumen (Colombatto *et al.*, 2006). However, in this study, the ruminal pH in sheep treated with the enzyme was only 0.3 lower than the control group, which suggests that, the clinical acidosis episodes were not high in the group feed with the enzyme.

The feed intake in the 64CSMG and the 45CSMG+E have a trend to be reduced (P=0.06) in a similar manner as that observed in dairy cows (Ferraretto *et al.*, 2011; Beckman and Weiss, 2005) that has not yet been observed in lambs receiving diets with different starch contents (Mendoza *et al.*, 2007). In evaluating the effects of amylolytic enzymes on intake, the addition of amylase did not affect intake in cows receiving a reduced starch diet. Ferraretto *et al.* (2011), and Crosby *et al.* (2006) did not find any differences in a high-grain diet when the amylase doses were increased (0.6 to 3 g/d); however, Buendía *et al.* (2003) reported a reduction in feed intake in sheep with 4 g/d of *Aspergillus niger* glucoamylase in a sorghum-based diet.

One experiment with different starch contents showed that feed conversion is improved with more grain (Mendoza *et al.*, 2005), and, for that reason, it was expected that the 64CSMG diet in this study (which was higher in grain) would result in a greater weight gain and better feed utilization efficiency; however, there were no differences shown. In bovines, there were similar results with high-grain diets (Stock *et al.*, 1990). However, in lambs, there is little information about this matter. In experiments with 12% differences in grain, there were no differences (Mota *et al.*, 2011; Lee *et al.*, 2012). However, the enzymes have shown the absence of an ADG in a form similar to the results reported by Rojo *et al.* (2005) and Mota *et al.* (2011). In contrast, Salem *et al.* (2011) reported a significant improvement in weight gain of lambs receiving a mixture of exogenous enzymes, which was associated with an increase in diet digestibility.

Experiments with amylolytic enzymes in lambs have not improved feed conversion (Buendía *et al.,* 2003; Mora *et al.*, 2002; Rojo *et al.*, 2005; Crosby *et al.*, 2006; Mota *et al.*, 2012). The 45CSMG+E had a ruminal pH 0.4 that was lower than the other treatments; for that reason, there is the possibility that the use of these enzymes may be causing sub-acute acidosis that prevents manifestation of a positive response; Lee *et al.* (2010) observed that buffer additions tended to improve responses in diets with amylolytic enzymes. It is important to consider that there are several factors involved in the response of these enzymes, such as enzyme origin, activity, dose, ruminal stability, grain type, grain treatment and ruminal pH, among others (Rojo *et al.*, 2007).

CONCLUSIONS

The inclusion of an enzyme in the diet with 45% grain demonstrated a trend toward negatively affecting (P=0.08) animal performance (ADG) and feed intake (P=0.06); however, the enzyme increased the *in sacco* and *in vitro* digestibility.

REFERENCES

AOAC. 1990. *Official Methods of Analysis of AOAC International*, 15th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Beckman, J.L. and Weiss, W.P. 2005. Nutrient digestibility of diets with different fibre-to-starch ratios when fed to lactating dairy cows. *Journal of Dairy Science,* 88: 1015-1023.

Buendía, R.G., Mendoza, M.G., Barcena, G.R., Ortega, C.M.E., Solís, H.J. and Lara, B.A. 2003. Efecto de la glucoamilasa de Aspergillus niger en la digestibilidad *in vitro* de maíz y sorgo y en la productividad de borregos. *Agrociencia*, 37: 317-322.

Colombatto, D., Moulda, F.L., Bhat, M.K. and Owen, E. 2003a. Use of fibrolytic enzymes to improve the nutritive value of ruminant diets. A biochemical and *in vitro* rumen degradation assessment. *Animal Feed Science and Technology,* 107: 201-209.

Colombatto D., Morgavi, D.P., Furtado, A.F. and Beauchemin, K.A. 2003b Screening of exogenous enzymes for ruminant diets, Relationship between biochemical characteristics and *in vitro* ruminal degradation. *Journal of Animal Science,* 81: 2628-2638.

Colombatto, D., Mould, F.L., Bhat, M.K. and Owen, E. 2006. Influence of exogenous fibrolytic enzyme level and incubation pH on the *in vitro* ruminal fermentation of alfalfa stems. *Animal Feed Science and Technology,* 137: 150-162.

Crosby, M.M., Mendoza, G.D., Melgoza, L.M., Bárcena, R., Plata, P.F. and Aranda, I.E. 2006. Effects of Bacillus licheniformis amylase on starch digestibility and sheep performance. *Journal of Applied Animal Research,* 30: 133-136.

Dhital, S., Shrestha, A.K. and Gidley, M.J. 2010. Relationship between granule size and *in vitro* digestibility of maize and potato starches. *Carbohydrate Polymers,* 82: 480-488.

Eun, J.S., Beauchemin, K.A. and Schulze, H. 2006a. Use of an *in vitro* fermentation bioassay to evaluate improvements in degradation of alfalfa hay due to exogenous feed enzymes. *Animal Feed Science and Technology,* 135: 315-328.

Eun, J.S., Beauchemin, K.A., Hong, S. H. and Bauer, M.W. 2006b. Exogenous enzymes added to untreated or ammoniated rice straw, Effects on *in vitro* fermentation characteristics and degradability. *Animal Feed Science and Technology,* 131: 86-101.

Eun, J.S., Beauchemin, K.A. 2007. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. *Animal Feed Science and Technology,* 132: 298-315.

Ferraretto, L.F., Shaver, R.D., Espineira, M., Gencoglu, H. and Bertics S.J. 2011. Influence of a reduced-starch diet with or without exogenous amylase on lactation performance by dairy cows. *Journal of Dairy Science,* 94: 1490-1499.

Gencoglu, H., Shaver, R.D., Steinberg, W., Ensink, J., Ferraretto, L.F., Bertics S.J., Lopes, J.C. and Akins M.S. 2010. Effect of feeding a reduced-starch diet with or without amylase addition on lactation performance in dairy cows. *Journal of Dairy Science,* 93: 723-732.

González, L.A., Manteca, X., Calsamiglia, S., Schwartzkopf-Genswein, K.S. and Ferret, A. 2012. Ruminal acidosis in feedlot cattle: Interplay between feed ingredients, rumen function and feeding behavior (a review). *Animal Feed Science and Technology,* 172: 66-79.

Gutierrez, C., Mendoza, G.D., Pinos, R.J.M., Ricalde, V.R., Aranda I.E. and Miranda L.A. 2005. Effect of storage time and processing temperature of grains with added amylolytic enzymes on *in situ* ruminal starch digestion. *Journal of Applied Animal Research,* 27: 39-44.

Herrera, S.R. and Huber, J.T. 1989. Influence of varying protein and starch degradabilities on performance of lactating cows. *Journal of Dairy Science,* 72: 1477-1483.

Herrera, H.J.G. and Barreras S.A. 2005. Análisis estadístico de experimentos pecuarios. 2nd Ed. Colegio de Postgraduados, Montecillo.

Htoon, A., Shrestha, A.K., Flanagan, B.M., Lopez-Rubio, A., Bird, A.R., Gilbert, E.P. and Gidley M.J., 2009. Effects of processing high amylose maize starches under controlled conditions on structural organisation and amylase digestibility. *Carbohydrate Polymers*, 75: 236-245.

Keulen, J.V. and Young, B.A. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *Journal of Animal Science,* 44: 282-287.

Klingerman, C.M., Hu W., McDonell, E.E., DerBedrosian, M.C. and Kung Jr. L. 2009. An evaluation of exogenous enzymes with amylolytic activity for dairy cows. *Journal of Dairy Science,* 92: 1050-1059.

Lee-Rangel, H.A., Pinos-Rodríguez, J.M., Mendoza, G.D., González, S.S., Montes, M.A., Trejo, A.S. and Jasso-Pineda Y. 2010. Effect of a ruminal buffer and exogenous amylolytic enzymes on growth and digestion in lambs fed high concentrate diets. *Journal of Applied Animal Research,* 37: 117-120.

Lee-Rangel, H.A., Mendoza, G.D. and González S.S., 2012. Effect of calcium propionate and sorghum level on lamb performance. *Animal Feed Science and Technology,* 177: 237-241.

Mendoza, M.G.D., Plata, P.F., Ramírez, M.M, Mejía, D.M.A., Lee R.H. and Bárcena G.R. 2007. Evaluación de alimentos integrales para el engorde intensivo de ovinos. *Revista Científica FCV-LUZ,* 15: 66-72.

Mora, J.G., Bárcena, G.R., Mendoza, M. G.D., González, M.S. and Herrera, H.J.G. 2002. Respuesta productiva y fermentación ruminal en borregos alimentados con grano de sorgo tratado con amilasas. *Agrociencia*, 36: 31-39.

Mota, N, Mendoza, G.D., Plata, F.X., Martínez, J.A., Lee, H., Rojo, R. and Crosby M.M. 2011. Effect of exogenous glucoamylase enzymes and reduction of grain level on lamb performance. *Journal of Applied Animal Research,* 39: 129-131.

Pinos, J.M., González, M.S., Mendoza, M.G.D., García, J.C., Miranda, L., De la Cruz, G. and De Lerma V. 2005. Efecto de enzimas fibrolíticas exógenas en la degradación *in vitro* de ingredientes alimenticios y en la producción de leche de vacas Holstein. *Interciencia*, 30: 752-757.

Pinos, R.J.M., González, M.S., Mendoza, G.D., Bárcena, G.R. and Martínez G.A., 2002. Análisis estadísticos de experimentos de digestibilidad *in vitro* con forrajes. *Interciencia*, 27: 143-146.

Rojo, R.R., Mendoza, M.G.D. and Crosby, G.M.M. 2001. Uso de la amilasa termoestable de Bacillus licheniformis en la digestibilidad '*in vitro*' del almidón de sorgo y maíz.*Agrociencia*, 35:423-427

Rojo, R.R., Mendoza, G.D., González, S., Landois, L., Bárcena R. and Crosby M.M. 2005. Effects of exogenous amylases from Bacillus licheniformis and Aspergillus niger on ruminal starch digestion and lamb performance. *Animal Feed Science and Technology,* 123-124: 655-665.

Rojo, R., Mendoza, G.D., Plata, F.X., Lara, A. and Bárcena R. 2007. Comparison of method of application on the effect of amylolytic enzymes on *in vitro* ruminal starch digestion. *Journal of Applied Animal Research,* 32: 81-84.

Salem, A.Z.M., El-Adawy, M., Gado, H., Camacho, L.M., González-Ronquillo, M., Alsersy, H. and Borhami B. 2011. Effects of exogenous enzymes on nutrients digestibility and growth performance in sheep and goats. *Tropical and Subtropical Agroecosystems*, 14: 867-874.

Schingoethe, D.J., Stegeman, G.A. and Treacher J.R. 1999. Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. *Journal of Dairy Science,* 82: 996-1003.

Stock, R.A., Sindt, M.H., Parrott, J.C. and Goedeken, F.K. 1990. Effects of grain type, roughage level and monensin level on finishing cattle performance. *Journal of Animal Science,* 68: 3441-3455.

Tilley, J.M.A. and Terry, R.A. 1963. A two-stage technique for the *in vitro* digestion of forage crops. *Journal of British Grassland Society*, 18: 104-111.

Van Soest, P.J., Robertson, J. and Lewis, B. 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3597.

Vanzant, E.S., Cochran, R.C. and Titgemeyer E.C. 1998. Standardisation of *in situ* techniques for ruminant feedstuff evaluation. *Journal of Animal Science,* 76: 2717-2729.

Weiss, W.P., Steinberg, W. and Engstrom, M.A. 2011. Milk production and nutrient digestibility by dairy cows when fed exogenous amylase with coarsely ground dry corn. *Journal of Dairy Science,* 94: 2492-2499.

Animal Nutrition and Feed Technology (2013) 13: 399-409

The Use of Exogenous Enzymes in Dairy Cattle on Milk Production and their Chemical Composition: A Meta-Analysis#

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ABSTRACT

Ortiz-Rodea, A., Noriega-Carrillo, A., Salem, A.Z.M., Castelan Ortega O. and González-Ronquillo, M. 2013. The use of exogenous enzymes in dairy cattle on milk production and their chemical composition: a meta-analysis. Animal Nutrition and Feed Technology, 13: 399-409.

We performed a meta-analysis to evaluate the effect of the addition of exogenous enzymes in ruminant feeding on milk production and chemical composition. We analysed the observations of 29 experiments, which included 52 treatments, 9 enzymes, and 1187 animals; with this information, we arranged a comprehensive database. The dose and study were used as experimental approaches. We observed that the addition of enzyme has no effect on the increment in milk yield production ($P=0.16$), fat content ($P=0.88$), lactose ($P=0.39$) or protein ($P=0.95$). The study showed that the variable milk yield is not a good parameter for determining with respect to the administration of exogenous enzymes ($R^2=0.001$). As a conclusion, it is necessary to reconsider the use of exogenous enzymes in domestic ruminants when the focus is to improve milk production and their chemical composition.

Key words: Enzymes, Meta-analysis, Milk yield, Ruminants

INTRODUCTION

Animal feeding is considered the major source of economic expenditures when referring to the production of milk and dairy products because they require high external inputs that allow us to keep elevated and constant production levels. Thus, milk production is not limited to dairy cattle only; also participating are domestic species such as sheep, goats, and in some regions such as Southeast Asia and Europe, native species such as the buffalo. Therefore the amount of feed required to maintain these productive farms, increases constantly and the agricultural surface area in the best of the cases is only maintained or it is decreasing. This is where the problem arises to maintain production and quality of milk yield and milk products with the least amount

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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of feed inputs. That is why it is necessary to make the nutrition of the animal more efficient, maximizing rumen activity and seeking to achieve sustainable production units. Thus ruminants exhibit endogenous enzymatic digestion, which allows them to obtain nutrients from food with complex structure (Pariza and Cook, 2010). Because of the benefits observed with these enzymes, several studies have tried to replicate this natural action mechanism by the addition of exogenous enzymes. The aim of this study was to conduct a meta-analysis to evaluate the effect of the addition of exogenous enzymes in feed for dairy cattle and its effects on the milk yield production and chemical composition

MATERIALS AND METHODS

Database development

The information search was focused on studies of exogenous enzymes supplementation in dairy cattle, and their effects on milk yield production and chemical composition to approach the number of studies recommended for this type of methodology (St Pierre, 2001). A database was conducted from experiments where both enzyme and dairy cattle, were specified from research published in scientific journals (Sauvant *et al.*, 2008). This included publications which were obtained from the ISI Web of Science database, Scopus, Redalyc, Routledge-Taylor and Francys Group, Science Direct and SpringerLink using the following keywords: exogenous enzymes, ruminants, milk yield, "enzymes and exogenous and ruminants," "enzymes and milk production," "enzymes and ruminants or dairy cattle." Additionally in the database, the following variables were recorded: number of animals in the study, basal diet, the enzyme used and its source, trade name of the enzyme, route of administration, dosage of enzyme (g/kg $LW^{0.75}$), milk yield production (kg/kg $LW^{0.75}$), and their chemical composition: protein, fat content, lactose (g/100g), and treatment duration (days).

We obtained a total of 29 studies, which included 52 experimental doses (Table 1) that provided the data for developing the basis of analysis. Sources of enzymes used in the studies were cellulase, xylanase, endoxylanase, amylase, protease, hemicellulase, exoglucanase, endoglucanase and glucanase. A total of 1187 animals were used for the studies analysed.

Statistical analysis

Analysis of the database was performed using a statistical approach meta-analysis (St-Pierre, 2001; Sauvant *et al.*, 2008). Using the MIXED procedure of SAS (version 9.2, SAS Institute Inc., 2008), the mixed model analysis used was $Y_{ij}=B_0 + B_1X_{ij} + s_i + b_iX_{ij} + e_{ij}$, where Y_{ij}=dependent variable, B_0=general intercept of all experiments (milk yield, fat, lactose and protein content), B_1=coefficient of linear regression coefficient of Y on X (exogenous enzyme), X_{ij}=value of the continuous predictor variable (exogenous enzyme dosage), s_i=random effect of study i, b_i=random effect of study i on X in study i, and e_{ij}=residual error not explained.

Table 1. Studies included in the meta-analysis of the effect of the addition of exogenous enzymes on milk yield production and chemical composition of dairy cattle.

Study	Reference	No. of animals	Basal feed	Enzyme	Enzyme source	Commercial enzyme product	Administration way	Enzyme dose (g/kg $LW^{0.75}$)	Trial duration (days)	kg $LW^{0.75}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
1	Arriola *et al.*, 2011	60	Forage, concentrate	Cellulase, xylanase and esterase	*Trichoderma longibrachiatum*	-	Sprayed on to TMR	0 and 3.4	63	118.19-120.32
2	Beauchemin *et al.*, 1999	4	Forage, concentrate	Cellulase, pectinase, xylanase	-	*Pro-Mote®*	Sprayed on to the TMR	0 and 2.50	23	122.14
3	Beauchemin *et al.*, 2000	6	Concentrate	Cellulase, endocellulase, endoglucanase, exoglucanase, xylanase		Natugrain® 33-L	Added to the concentrates	0, 1.22 and 3.67	21	128.88-129.33
4	Bernard et al, 2010	44	Corn silage and alfalfa or T85 haylage	Celllulase	-	*Promote ®N.E.T.*	Applied to TMR	0 and 0.006	56	127.78-137.20
5	Bowman *et al.*, 2002	8	Forage, concentrate mixture	Cellulase, xylanase	-	*Promote ®N.E.T.*	Added to concentrate and premix, pelleted	0 and 0.0015	28	128.58
6	DeFrain *et al.*, 2005	24	Hay, haylage, concentrate	Endoglucanase, xylanase	*Saccharomyces cerevisiae* and *Aspergillus oryzae*	-	Added to TMR	0 and 0.017	21	114.04-115.27
7	Dhiman *et al.*, 2002	50	Hay, silage, concentrate	Cellulase, xylanase	-	*Bovizyme®*	Applied to the forage	0, 0.002 and 1.3	270	128.91
8	Elwakeel *et al.*, 2007	24	Silage, hay, concentrate	Fibrolytic	-	-	Mixed in ration	0 and 5-15	56	122.59-124.55

Table 1. Contd. .....

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
9	Eun *et al.*, 2005	8	Forage, concentrate	Protease	*Bacillus licheniformis*	*Protex® 6L*	Sprayed on to the pelleted supplement	0 and 1.25	21	134.63
10	Ferraretto *et al.*, 2011	45	Forage	Amylase	-	*Ronozyme RumiStar®*	Added to concentrate	0 and 3.8	70	139.72-140.87
11	Gado *et al.*, 2009	20	Corn silage, concentrate	Amylase, cellulase, protease, xylanase	Anaerobic ruminal bacteria	*ZADO®*	Added to TMR	0 and 40	84	105.74
12	Gencoglu *et al.*, 2010	36	Corn silage, alfalfa silage, concentrate	Amylase	-	*Ronozyme RumiSta®r*	Sprinkled on to concentrate	0 and 1250	84	134.92-135.94
13	Holtshausen *et al.*, 2011	60	Silage, hay, concentrate	Endoglucanase, xylanase	-	*Econase® RDE*	Added to TMR	0 and 0.5-1	70	114.04-115.27
14	Klingerman *et al.*, 2009	28	Concentrate, silage, hay, haylage	Amylase	*Saccharomyces cerevisiae, Aspergillus oryzae, A. oryzae*	*7B enzyme formulation*	Sprayed on to concentrate	0, 0.88 and 4.40	21	143.03-144.18
15	Knowlton *et al.*, 2002	34	Forage, concentrate	Celllulase	Commercial preparation from fungal extracts	-	Added to TMR	0 and 0.204	28	117.73-126.35
16	Knowlton *et al.*, 2007	24	Corn silage, alfalfa silage	Cellulase, phytase	Fungal extracts	-	Mixed with a corn grain carrier	0 and 0.297	31	120.59-121.43
17	Kung *et al.*, 2000	60	Corn silage, alfalfa, pelleted concentrate	Cellulase, hemicellulase, xylanase	*Trichoderma longibrachiatum*	-	Sprayed on to forages	0, 2 and 10	84	119.10-129.77
18	Kung *et al.*, 2002	30	Corn silage, alfalfa, pelleted concentrate	Cellulase, xylanase	-	-	Sprayed on to forages	0 and 10	84	129.03-129.62

Table 1. Contd. .....

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
19	Lewis *et al.*, 1999	70	Alfalfa silage, hay, concentrate	Cellulase, xylanase	-	*Cornzyme®*	Applied to the forage	0, 1.25 and 5.0	21 and 112	121.23-131.54
20	Miller *et al.*, 2008	72	Concentrate	-	*Trichoderma longibrachiatum*	*Roxazyme® G2 Liquid*	Applied to barley or sorghum	0, 2.15 and 4.3	75	111.86-112.80
21	Pinos-Rodríguez *et al.*, 2005	40	Forage, concentrate	Xylanase	*A. niger y T. viridae*	*Fibrozyme®*	Added to concentrate mixture	0 and 1.3	120	121.23
22	Reddish *et al.*, 2007	24	Silage, hay, concentrate	Cellulase, xylanase	-	-	Mixed by hand into the TMR	0 and 0.016	21	119.86
23	Rode *et al.*, 1999	20	Silage, hay, concentrate	Cellulase, xylanase	-	*Pro-Mote®*	Diluted and added to concentrate	0 and 1.3	84	126.65-131.40
24	Sutton *et al.*, 2003	4	Silage, concentrate	Endoglucanase, xylanase	*Trichoderma longibrachiatum*	-	Sprayed on to the TMR	0 and 2	35	124.7
25	Vicini *et al.*, 2003	233	Silage, hay, concentrate	Fibrolytic	*Trichoderma longibrachiatum*	-	Sprayed on forage and mixed ration	0, 0.00038 and 2	84	121.23
26	Weiss *et al.*, 2011	28	Silage, concentrate	Amylase	-	*Ronozyme RumiStar ®*	Added to concentrate mix	0 and 0.125	98	123.35-127.54
27	Yang *et al.*, 1999	4	Concentrate, silage, hay	Cellulase, xylanase	-	*Pro-Mote®*	Sprayed on to hay	0, 1 and 20.8	21	124.10-125.15
28	Yang *et al.*, 2000	43	Concentrate, hay, silage	Cellulase, xylanase	*Trichoderma longibrachiatum*	-	Sprayed on to TMR or applied to concentrate	0 and 0.5	105	136.09
29	Zheng *et al.*, 2000	48	Forage, concentrate	Xylanase	-	*Bovizyme®*	Sprinkled on to the forage	0 and 1.25	126	122.74-130.21

TMR: Total Mixed Ration.

The variable in the study was determined in the level CLASS. This presented no quantitative data, and we determined the structure for unstructured matrix of the covariance (TYPE=UN), and this was specified in the random model to avoid positive correlation between intercepts and slopes. In addition, we calculated the standard deviation, the P value, the standard error mean (SEM) and the coefficient of determination. In reference to the graphic representation of the results of the meta-analysis, an adjustment of the response variables was made, taking into account the random effect of the study. Similarly, variables were standardized in relation to the metabolic live weight ($LW^{0.75}$) to avoid variation between studies.

RESULTS

The mixed analysis showed 67 enzyme dosages with a range of 0.0002 to 3.48 g/kg $LW^{0.75}$. However, for the variable response milk yield production, there were no differences (P>0.16) between doses. Also, the coefficient of determination was lower, between the enzyme doses and the milk yield production response as shown in Fig. 1.

The chemical composition of milk showed no significant differences (P=0.88) for fat component (Fig. 2), presenting a low coefficient of determination, which indicates the poor relationship between the administration of the enzyme and milk fat composition, with a negative effect. Similar results were obtained when the component lactose was determined (Fig. 3), (P=0.39). Also there were no differences (P=0.95) in the crude protein content in milk (Fig. 4) due to the addition of enzymes to the feed.

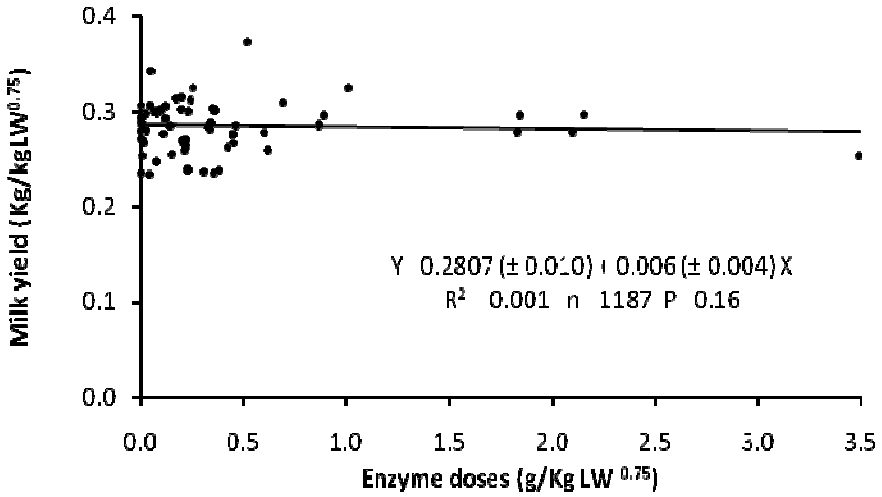

Fig. 1. Effect of the addition of exogenous enzymes (g/kg $LW^{0.75}$) on milk yield production (kg/kg $LW^{0.75}$) in dairy cattle.

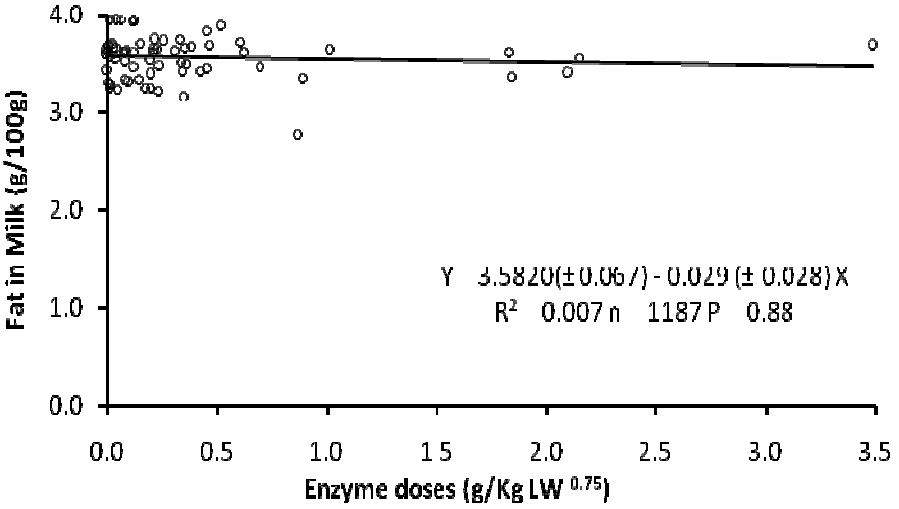

Fig. 2. Effect of exogenous enzymes intake (g/kg $LW^{0.75}$) on the composition of milk fat (g/100g) in dairy cattle.

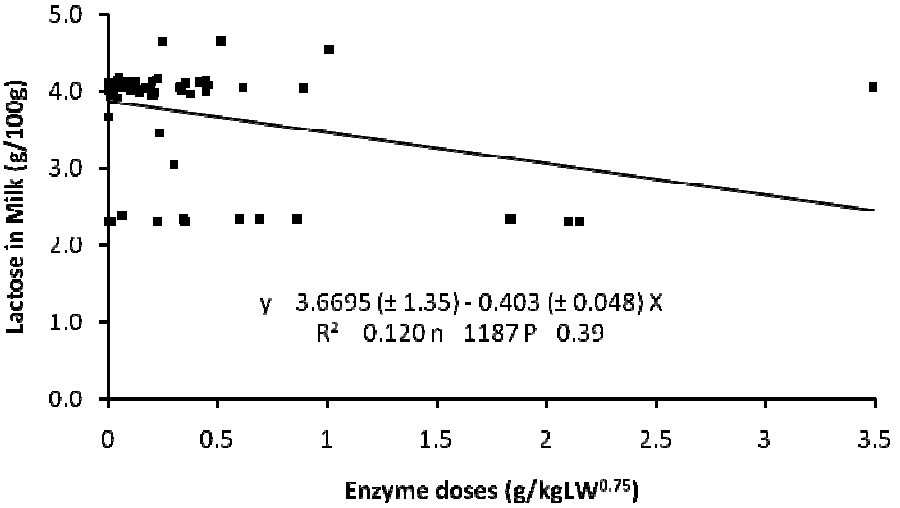

Fig. 3. Effect of exogenous enzymes intake (g/kg $LW^{0.75}$) on the content of lactose in dairy cattle milk (g/100g).

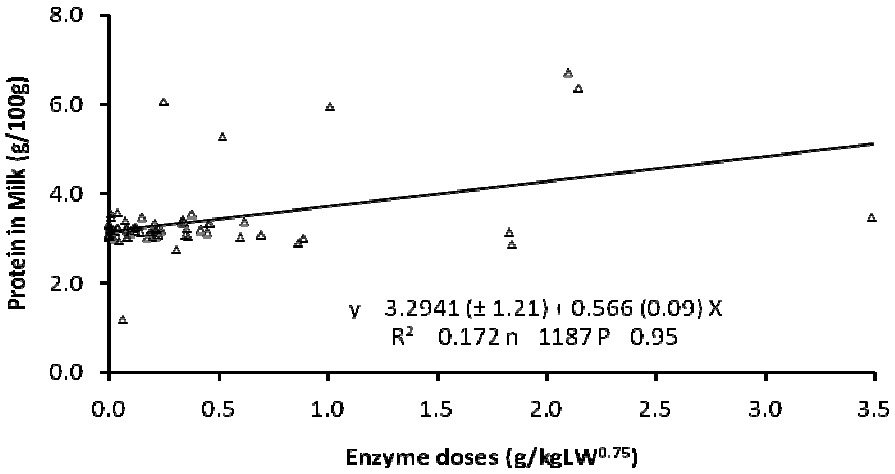

Fig. 4. Effect of exogenous enzymes intake (g/kg $LW^{0.75}$) on milk protein content (g/100g) in dairy cattle.

DISCUSSION

The analysis indicates there was no effect of the addition of exogenous enzymes in the animal feed offered to the variables on milk yield production; these results are consistent with Flores *et al.* (2008), Bowman *et al.* (2002), Beauchemin *et al.* (1999) and Rode *et al.* (1999). In contrast, studies by Titi and Stella found an effect when utilizing enzymes, with an increase in the amount of milk yield produced by goats fed with supplementation of yeast culture. Similar results are shown by Kung *et al.* (2000), Lewis *et al.* (1999), Zheng *et al.* (2000) and Yang *et al.* (1999; 2000) in dairy cattle. The results that show no effect when supplemented by enzymes can be influenced by the dose and type of enzyme. Kung *et al.* (2000) suggests that overdose of enzymes causes decreased chewing due to an increase in the digestibility of the feed; this, in turn, decreases the production of saliva, ruminal pH and thus generates less fiber digestion, resulting in less amount of milk yield produced; meanwhile Treacher *et al.* (1996) suggests that excessive doses of enzymes affect the ruminal micro-organisms adhering to the substrate and also promote the release of anti-nutritional factors as secondary compounds, thereby reducing the microbial digestion. Moreover the combination of exogenous enzymes (Morgavi *et al.*, 2001) is capable of withstanding the ruminal and intestinal proteolysis, such is the case of compounds derived from *Trichoderma longibrachiatum* fungus.

The analysis showed no effect in the milk fat content by the addition of enzyme, however, the slope was negative, indicating that a higher enzyme doses diminish the milk fat content; this effect coincides with Kung *et al.* (2000), Rode *et al.* (1999), and Stella *et al.* (2007) who found a decrease in the milk fat content of animals that were

fed various doses of enzyme. Meanwhile Beauchemin *et al*. (1999), Flores *et al*. (2008), and Titi *et al*. (2004) indicate no effect in the milk fat content by the addition of enzyme. In contrast Bowman *et al*. (2002) found an increased milk fat component when supplemented by enzyme in the food of dairy cattle. The lactose content was not affected by the addition of exogenous enzymes; these results are consistent with Beauchemin *et al*. (1999) and Rode *et al*. (1999), but differ from Bowman *et al*. (2002) who found an increased lactose content. The milk protein content coincides with Flores *et al*. (2008), Titi *et al*. (2004), and Rode *et al*. (1999) who indicate no effect on the amount of enzyme protein in milk. The absence of increased protein content in milk can be caused by changes in protein metabolism in the rumen; studies by Yang *et al*. (1999) mention that the fibrolytic enzymes increase the degradation of dietary protein in the rumen, which in turn increases the synthesis of microbial crude protein. Meanwhile Rode *et al*. (1999) found that the increase in the endogenous protein is due to the catalytic effect of enzymes on the exogenous protein, causing insufficient protein levels on step. This greater amount of imbalance and microbial protein of lower protein content of the input step has an effect on amino acids in milk, which according with Chalupa *et al*. (2000) is 50 to 55% of amino acids originating from microbial protein and from 45 to 50% amino acids provided by the rumen undegradable protein. On the other hand Kung *et al*. (2000) found a negative effect on the protein with the inclusion of enzymes; on the contrary Bowman *et al*. (2002), showed an increase in this parameter.

CONCLUSION

The parameter milk yield production and their components of fat, lactose and protein have no effect to the administration of exogenous enzymes. It is necessary to reconsider its use in ruminants when the aim is to increase milk yield production and their chemical composition.

ACKNOWLEDGMENT

Mr. Arturo Ortiz was granted for a CONACyT fellowship during his studies in the Autonomous University of the State of Mexico as a Doctorate in the PCARN program. Miss Alejandra Noriega was granted for a CONACyT fellowship during her studies in the Autonomous University of the State of Mexico, as a Specialist in the Sheep Production Specialization program. We also thank Miss Liz Hopper, LTC-University of North Texas for the critical review of the present manuscript.

REFERENCES

Arriola, K.G., Kim, S.C., Staples, C.R. and Adesogan, A.T. 2011. Effect of fibrolytic enzyme application to low and high-concentrate diets on the performance of lactating dairy cattle. *Journal of Dairy Science*, 94, 832-841

Beauchemin, K.A., Yang, W.Z. and Rode L.M. 1999. Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. *Journal of Dairy Science,* 82: 378-390.

Beauchemin, K.A., Rode, L.M., Maekawa, M., Morgavi, D.P. and Kampen, R. 2000. Evaluation of a nonstarch polysaccharidase feed enzyme in dairy cow diets. *Journal of Dairy Science*, 83: 543-553.

Bernard, J.K., Castro, J.J., Mullis, N.A., Adesogan, A.T., West, J.W. and Morantes, G. 2010. Effect of feeding alfalfa hay or Tifton 85 bermudagrass haylage with or without a cellulase enzyme on performance of Holstein cows. *Journal of Dairy Science,* 93: 5280-5285.

Bowman, G.R., Beauchemin, K.A. and Shelford, J.A. 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. *Journal of Dairy Science*, 85: 3420-3429.

Chalupa, W. and Sniffen, C.J. 2000. Balancing rations for milk components. *Asian-Australasian Journal of Animal Science*, 13 (Suppl.): 388-396.

De Frain, J.M., Hippen, A.R., Kalscheur, K.F. and Tricarico, J.M. 2005. Effects of dietary α-amylase on metabolism and performance of transition dairy cows. *Journal of Dairy Science*, 88: 4405-4413.

Dhiman, T.R., Zaman, M.S., Gimenez, R.R., Walters, J.L. and Treacher, R. 2002. Performance of dairy cows fed forage treated with fibrolytic enzymes prior to feeding. *Animal Feed Science and Technology,* 101: 115-125.

Elwakeel, E.A., Titgemeyer, E.C., Johnson, B.J., Armendariz, C.K. and Shirley, J.E. 2007. Fibrolytic enzymes to increase the nutritive value of dairy feedstuffs. *Journal of Dairy Science*, 90: 5226-5236.

Eun, J.S. and Beauchemin, K.A. 2005. Effects of a proteolytic feed enzyme on intake, digestion, ruminal fermentation, and milk production. *Journal of Dairy Science*, 88: 2140-2153.

Ferraretto, L.F., Shaver, R.D., Espineira, M., Gencoglu, H. and Bertics S.J. 2011. Influence of a reduced-starch diet with or without exogenous amylase on lactation performance by dairy cows. *Journal of Dairy Science*, 94: 1490-1499.

Gado, H.M., Salem, A.Z.M., Robinson, P.H. and Hassan, M. 2009. Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Animal Feed Science and Technology,* 154: 36-46.

Gencoglu, H., Shaver, R.D., Steinberg, W., Ensink, J., Ferraretto, L.F., Bertics, S.J., Lopes, J.C. and Akins M.S. 2010. Effect of feeding a reduced-starch diet with or without amylase addition on lactation performance in dairy cows. *Journal of Dairy Science*, 93: 723-732.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M. and Beauchemin, K.A. 2011. Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. *Journal of Dairy Science*, 94: 899-907.

Klingerman, C.M., Hu W., McDonell E.E., DerBedrosian M.C. and Kung L. Jr. 2009. An evaluation of exogenous enzymes with amylolytic activity for dairy cows. *Journal of Dairy Science*, 92: 1050-1059.

Knowlton, K.F., McKinney, J.M. and Cobb, C. 2002. Effect of a direct-fed fibrolytic enzyme formulation on nutrient intake, partitioning, and excretion in early and late lactation holstein cows. *Journal of Dairy Science*, 85: 3328-3335,

Knowlton, K.F., Taylor M.S., Hill, S.R., Cobb, C. and Wilson, K.F. 2007. Manure nutrient excretion by lactating cows fed exogenous phytase and cellulase. *Journal of Dairy Science*, 90: 4356-4360.

Kung, L.Jr., Treacher, R.J., Nauman, G.A., Smagala, A.M., Endres, K.M. and Cohen, M.A. 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. *Journal of Dairy Science*, 83: 115-122.

Kung, L.Jr., Cohen, M.A., Rode, L.M. and Treacher, R.J., 2002. The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ratio to lactating dairy cows. *Journal of Dairy Science*, 85: 2396-2402.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I. and Treacher R.J. 1999. Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. *Journal of Dairy Science*, 82: 611-617.

Miller, D.R., Granzin, B.C., Elliott, R. and Norton, B.W. 2008. Effects of an exogenous enzyme, Roxazyme® G2 Liquid, on milk production in pasture fed dairy cows. *Animal Feed Science and Technology,* 145: 194-208.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., McAllister, T.A., Iwaasa, A.D., Wang, Y. and Yang, W.Z. 2001. Resistance of feed enzymes to proteolytic inactivation by rumen micro-organisms and gastrointestinal proteases. *Journal of Animal Science*, 79: 1621-1630.

Pariza, M.W. and Cook, M. 2010. Determining the safety of enzymes used in animal feed. *Regulatory Toxicology and Pharmacology,* 56: 332-342.

Pinos-Rodríguez, J.M., González, S., Mendoza, G., García, J.C., Miranda, L., De la Cruz, G.A. and De Lerma, V. 2005. Efecto de enzimas fibrolíticas exógenas en la degradación *in vitro* de ingredientes alimenticios y en la producción de leche de vacas Holstein. *Interciencia,* 12: 752-757.

Reddish, M.A. and Kung, L. Jr. 2007. The effect of feeding a dry enzyme mixture with fibrolytic activity on the performance of lactating cows and digestibility of a diet for sheep. *Journal of Dairy Science*, 90: 4724-4729.

Rode, L.M., Yang, W.Z. and Beauchemin, K.A. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. *Journal of Dairy Science*, 82: 2121-2126.

SAS. 2008. *SAS/STAT Software, Version 9.2.* SAS Institute Inc., Cary, NC, USA.

Sauvant, D., Schmidely, P., Daudin, J.J. and St-Pierre, N.R. 2008. Meta-analyses of experimental data in animal nutrition. *Animal,* 2: 1203-1214.

St-Pierre, N.R. 2001. Integrating quantitative findings from multiple studies using mixed model methodology. *Journal of Dairy Science*, 84: 741-755.

Sutton, J.D., Phipps, R.H, Beever, D.E., Humphries, D.J., Hartnell, G.F., Vicini, J.L. and Hard, D.L. 2003. Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in holstein-friesian cows. *Journal of Dairy Science*, 86: 546-556.

Treacher, R.J. and Hunt, C.W. 1996. Recent developments in feed enzymes for ruminants. Pacific Northwest Nutrition Conference. Seattle, W.A.

Vicini, J.L., Bateman, H.G., Bhat, M.K., Clark, J.H., Erdman, R.A., Phipps, R.H., Van Amburgh, M.E., Hartnell, G.F., Hintz, R.L. and Hard, D.L. 2003. Effect of feeding supplemental fibrolytic enzymes or soluble sugars with malic acid on milk production. *Journal of Dairy Science*, 86: 576-585.

Weiss, W.P., Steinberg, W. and Engstrom, M.A. 2011. Milk production and nutrient digestibility by dairy cows when fed exogenous amylase with coarsely ground dry corn. *Journal of Dairy Science*, 94: 2492-2499.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 1999. Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. *Journal of Dairy Science*, 82: 391-403.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science*, 83: 2512-2520.

Zheng, W., Schingoethe, D.J., Stegeman, G.A., Hippen, A.R. and Treacher, R.J. 2000. Determination of when during the lactation cycle to start feeding a cellulase and xylanase enzyme mixture to dairy cows. *Journal of Dairy Science*, 83: 2319-2325.

Animal Nutrition and Feed Technology (2013) 13: 411-422

Effects of Exogenous Enzymes and *Salix babylonica* L. Extract on Cellular Immune Response and its Correlation with Average Daily Weight Gain in Growing Lambs#

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ABSTRACT

Rivero, N., Salem, A.Z.M., Ronquillo, M.G., Cerrillo-Soto, M.A., Camacho, L.M., Gado, H. and Peñuelas, C.G. 2013. Effects of exogenous enzymes and Salix babylonica L. extract on cellular immune response and its correlation with average daily weight gain in growing lambs. Animal Nutrition and Feed Technology, 13: 411-422.

The aim of this study was to determine the effects of exogenous enzyme (EZ) and *Salix babylonica* L. (SB) extract on cellular immune response, and its correlation with average daily weight gain (ADG). Twenty Suffolk lambs, 6-8 months of age and average live weight of 24±0.3 kg, were used in a trial which lasted 60 days. The lambs were distributed into 4 groups of 5 lambs each and housed in individual 1.5x1.5 m cages in a completely randomized design. The treatments were: (i) Control; lambs consuming basal diet (BD) only; (ii) EZ; lambs consuming BD plus 10g of EZ (ZADO®); (iii) SB; lambs consuming BD plus 30 mL of SB, and (iv) EZSB; lambs consuming BD plus 10g EZ and 30 mL of SB. Blood samples were collected on days 0, 15, 30, 45 and 60 and analysed for helper T lymphocytes, cytotoxic T limphocytes, granulocytes and monocytes by flow cytometry. Treatments had no effect on parameters measured, but day of sampling had linear and cubic effects on helper T lymphocytes, granulocytes and monocytes ($P<0.01$) and cubic effects on cytotoxic T lymphocytes ($P<0.01$). The results suggest that EZ and SB have immunosuppressant effects in the first 15 days, after this effect were immunosuppressive on cytotoxic T lymphocytes and granulocytes, for monocytes the effect was immunostimulant. No there were correlation between ADG and cellular immune response in this experiment.

Key words: Exogenous enzyme, *Salix babylonica* L. extract, Immune response, Performance, Lambs.

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

There is a complex tripartite interaction between malnutrition, immune system and infectious diseases (Mitchell *et al.*, 2003). Deficiency of macro- and micro-nutrients causes dysfunction of the immune system (Mitchell *et al.*, 2003; Chandra, 2004; Wintergerst *et al.*, 2007). These deficiencies increase the vulnerability of animals to disease which is exacerbated by malnutrition both altering defense mechanisms such as anatomical barriers, cellular and humoral immune responses against antigens (Calder and Kew, 2002; Maggini *et al.*, 2007). Thus, the animal is not capable to initiate an immune response and the infection might not be controlled (Erickson *et al.*, 2000; Enwonwu, 2006) leading the animal to under-express their productive potential.

In order to promote a more efficient use of macro and micro-nutrients contained in the diet, feed additives such as pre- and probiotics, enzymes and exogenous ionophores and fodder tree extracts have been examined in previous studies (Salem *et al.*, 2010; Salem *et al.*, 2011a; Chung *et al.*, 2012). Although there is evidence that such feed additives can directly or indirectly maintain the immunocompetence of the animal, namely by stimulating cells of the immune system or providing nutrients for these cells to function normally (Nayak, 2010; Owusu-Asiedu *et al.*, 2010).

The activity of ZADO® which is a commercial endogenous enzyme (EZ), has proved to increase the digestibility of DM, CP, NDF, ADF and the concentration of volatile fatty acids in rumen, and thus, resulting in improvements in animal productivity by increasing feed intake, ADG, feed efficiency and milk production (Arriola *et al.*, 2011; Gado *et al.*, 2011). Addition of EZ increased forage energy utilization which may have an indirect effect on the immune system under the assumptions of immune nutrition which are based in adequate utilization of dietary nutrients to promote maintenance of immunocompetence (Keith and Jeejeebhoy, 1997).

There are different types of possible measurements to investigate relationships between nutrition and immune response. Phenotyping and enumeration of immune cells by flow cytometry has proven to be accurate allowing the comparison of cell ratios and analysis of cell counts (Mitchell *et al.*, 2003). The objective was to determine effects of exogenous enzyme and *S. babylonica* L. extract on cellular immune response and to evaluate if it correlates with average daily weight gain in lambs.

MATERIALS AND METHODS

This study was conducted at the experimental farm unit of the Faculty of Veterinary Medicine of the Autonomous University of Mexico State. The handling of animals was performed according to international bioethical standards and NOM-062-ZOO-1999 (SAGARPA, 1999).

Animals and treatments

Twenty Suffolk lambs, 6-8 months of age and average live weight of 24 ± 0.3 kg, were used. Lambs were housed in individual 1.5×1.5 m cages in a completely

randomized design and the experiment was conducted for a period of 60 days. After 2 weeks of adaptation to the basal diet of 70% corn silage and 30% commercial concentrate (Purina®, Cuautitlan, Mexico) which was formulated to meet nutrient requirements (NRC, 1985), the lambs were weighed and randomly distributed into 4 groups of 5 lambs. The treatments were: (i) Control: basal diet of concentrate and corn silage; (ii) EZ: basal diet plus 10 g/day of exogenous enzyme preparations (ZADO®, Cairo, Egypt); (iii) SB: basal diet plus 30 mL/day of *Salix babylonica* L. extract, and (iv) EZSB: basal diet plus 10 g/day exogenous enzymes and 30 mL of *S. babylonica* L. extract. The daily dose of SB was given orally before the morning feeding; the EZ was mixed with 200 g of concentrate and offered 1 h before the rest to corn silage and concentrate was provided. The chemical composition of the basal diets is shown in Table 1.

Table 1. Chemical composition of the basal diet (commercial concentrate and corn silage) and levels of secondary metabolites (g/kg DM) in the *S. babylonica* L. extract

	Concentrate	Corn silage	*S. babylonica* L. extract
Chemical composition			
Dry matter[1]	880	360	
Organic matter	325	684	
Crude protein	157.1	106.2	
Ether extract	119.8	85.2	
Neutral detergent fiber	160.4	444.8	
Acid detergent fiber	27.7	111.2	
Lignin	7.6	18.0	
Ingredients of concentrate g/kg			
Corn grain flacked	200		
Corn grain cracked	260		
Sorghum grain	154		
Molasses sugar cane	100		
DDG	100		
Soya bean meal	96		
Weath bran	70		
$NaCOO_3$	10		
Mineral mixture[2]	10		
Secondary metabolites			
Total phenolics	-	-	16.4
Saponins	-	-	5.4
Aqueous fraction[3]	-	-	76.3

[1]DM expressed as g/kg fresh silage; [2]Mineral mixture: Ca, 190 g/d; P, 115 g/d; Mg, 63 g/d; Cl, 167 g/d; K, 380 g/d; Na, 70 g/d; S, 53 g/d; Co, 3.3 mg/d; Cu, 197 mg/d; Fe, 360 mg/d; Mn, 900 mg/d; Se, 2 mg/d; Zn, 810 mg/d; vitamin A, 940 (1000 IU/d); vitamin D, 165 (1000 IU/d); vitamin E, 374 (1000 IU/d); [3]The aqueous fraction contains lectins, polypeptides and starch (Cowan, 1999).

ZADO® is a powdered multi-enzyme commercially available feed additive produced from *Ruminococcus flavefaciens* by the Academy of Scientific Research and Technology in Egypt (Patent No.: 22155, Cairo, Egypt). Prior to the study, the mixture was assayed for several enzymatic activities and found to contain (/g of enzyme preparation) 7.1 units of endoglucanase, 2.3 units of xylanase, 61.5 units of α- amylase and 29.2 units of protease activity (Gado *et al.*, 2011).

In order to prepare silage, whole corn plants (at ~70% moisture) were chopped into 1 to 2 cm pieces using a forage chopper. Silage was accomplished in a flat 10 t silo and after 2 months, it was offered for feeding.

Preparation of extract

The *S. babylonica* L. extract was prepared as described by Salem *et al.* (2011b). Briefly, fresh leaves of *S. babylonica* L. were collected randomly from several young and mature trees (minimum 5 different trees) in autumn and chopped (1 to 2 cm) and immediately extracted in the proportion of 1 g leaf per 8 mL of solvent mixture, which contained 10 mL methanol (99.8/100, analytical grade, Fermont®, Monterrey, Mexico), 10 mL ethanol (99/100, analytical grade, Fermont®, Monterrey, Mexico) and 80 mL distilled water. Leaves were soaked and incubated in this solvent in the laboratory at 25 to 30°C for 48 to 72 h in closed flasks. After incubation, all flasks were incubated in a water bath at 39°C for 1 h and then immediately filtered and the filtrate collected and stored at 4°C for further use.

Sampling and measurements

Five ml of peripheral blood were withdrawn from each animal via jugular venipuncture into Heparin vacutainer tubes (BD tube, Monterrey, Mexico) kept in ice on days 0, 15, 30, 45 and 60 of the experiment. Blood was analysed to determine immune phenotypes by flow cytometry. The immune phenotypes determined were helper T lymphocytes (Ab mouse anti sheep CD4RPE/MCA2213PE), cytotoxic T lymphocytes (Ab mouse anti sheep CD8FITC/MCA2216F, D.F, Mexico), granulocytes (Ab mouse anti bovine CD11bFITC/MCA1425F,.D.F, Mexico) and monocytes (Ab mouse anti human CD14RPE/MCA1568PE, D.F, Mexico) with antibodies AbDSerotec®.

The animals were weighed on the day of sampling, every 15 days. The feed offered and rejected was weighed every day. Two samples of concentrate, silage and SB extract were collected weekly and stored at -20°C for later chemical analysis. Additional samples by type (*i.e.,* concentrate, silage, extract) were pooled and stored for further analysis.

Processing of samples

Each sample was processed to determine the four immune phenotypes, initially the cellular gradient was obtained using Ficoll (Limphoprep® BD) to recover white

blood cells as lymphocytes (T helper and cytotoxic), granulocytes (neutrophils, eosinophils and basophils) and monocytes (macrophages) (Macey, 2007).

Gradient

Blood sample and PBS 1 x were used to make a 1: 1 dilution. This mixture was placed in 3 mL of cold lymphoprep®, centrifuged at 1200 rpm at 4°C for 20 min, then the collected cells were placed in 2 mL of PBS 1x and centrifuged again at 1500 rpm at 4°C for 5 min (Macey, 2007).

Sample staining

After the cell gradient was processed, 20 μL of each sample was placed in a 96 well plate and centrifuged at 1500 rpm for 5 min. The supernatant was removed and 50 μL of the adequate antibody was incorporated (helper T lymphocytes, cytotoxic T lymphocytes, granulocytes, monocytes). After 30 min at 4°C incubation, 100 μL of PBS 1x was added, centrifuged again and the supernatant removed, while a secondary antibody was added and incubated for 30 min at 4°C. Then a final wash with PBS 1x was performed. The readings were conducted in a flow cytometer (FacsCalibur) (Macey, 2007).

Chemical analyses and assays

Samples of concentrate and silage were analysed for DM (#934.01), ash (#942.05), N (#954.01) and EE (#920.39) according to AOAC (1997) and Van Soest *et al.* (1991). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin (sa) (AOAC, 1997; #973.18) were analysed using an ANKOM 200 Fibre Analyzer Unit (ANKOM Technology Inc., Macedon, NY, USA). The NDF was assayed without use of an alpha amylase but with sodium sulphite. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites were determined using 10 mL of extract liquor and fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont®, Monterrey, Mexico) to determine total phenolics by drying and to quantify the total phenolics layer in the funnel. After total phenolics separation, a double volume of n-butanol (99.9/100, analytical grade, Fermont®, Monterrey, Mexico) was added to fractionate saponins (Ahmed *et al.*, 1990). The remaining solution was considered to be the aqueous fraction which contains the other secondary metabolites, lectins, polypeptides and starch (Cowan, 1999; Table 1).

Endoglucanase activity was assayed by liberating glucose from carboxymethyl cellulose, which was determined calorimetrically using alkaline copper reagent as described by Robyt and Whelan (1972). One unit of endoglucanase catalyzes liberation of one mmol of glucose/min from sodium carboxymethyl cellulose at 40°C and pH 4.5. The α-amylase was assayed by its ability to produce reducing groups from starch, which were measured by reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955). One unit of

α-amylase catalyzes liberation of one mmol of reducing groups/min from soluble starch at 25°C and pH 6.0, calculated as maltose equivalents. Protease activity was determined by hydrolysis of dimethyl casein (DMC) and liberated amino acids were determined using 2,4,6-trinitrobenzene sulfonic acid (Lin *et al.*, 1969). One DMC-U catalyzes cleavage of one mmol of peptide bond/min from DMC at 25°C and pH 7.0 expressed in terms of newly formed terminal amino groups. Xylanase catalyzes hydrolysis of xylan from oat spelt, and the reducing groups liberated were determined using alkaline copper reagent (Robyt and Whelan, 1972). One unit catalyzes liberation of one mmol reducing groups per hour from xylan at 37°C and pH 5.5, expressed as xylose equivalents.

Statistical analyses

Data related to immunological parameters were analysed using the MIXED procedure of SAS (2002) with repeated measures (Littell *et al.*, 1998). The structure of the variance-covariance error matrix employed was unstructured, based on Bayesian criteria observed with several alternative structures. Terms in the model were diet (*i.e.,* control, SB, EZ, EZSB), days of sampling (*i.e.,* 0, 15, 30, 45 and 60 of the experiment) and its linear, quadratic and cubic effects. The repeated term was sampling days, with lamb within diet as the subject. Results are reported in Tables and in text with their respective standard error of the mean. Tests of simple effects were used to partition interaction effects by diet in order to test effects of period separately for each diet using SAS. Significant differences between treatment means and time were assessed using Tukey's test at $P<0.05$ level. Additionally, correlation analyses between ADG and each of the four immunological parameters (*i.e.,* helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes) for treatment and day of sample were performed using PROC CORR of SAS.

RESULTS

Effect of EZ on immunological parameters

Treatment resulted in no differences on the four immunological parameters measured (Table 2). Addition of this additive resulted in no effects on DMI and ADG (Table 2). Similarly, no correlations between ADG and each of the immunological parameters such as helper T lymphocytes, cytotoxic T lymphocytes, granulocytes and monocytes were registered.

Effect of SB extract on immunological parameters

There were no differences among treatments due to incorporation of SB to the diets of lambs. The values of helper T lymphocytes and cytotoxic T lymphocytes increased in lambs offered SB compared to the control. Granulocytes and monocytes, however, decreased in lambs fed SB compared to the control (Table 2). The DMI and ADG were unaffected by the intake of *S. babylonica* extract (Table 2). The ADG was not correlated with helper T lymphocytes or cytotoxic T lymphocytes. The same trend

Table 2. Effects of exogenous enzyme preparation (EZ) and *S. babylonica* L. (SB) extract as well as their mixture (EZSB) on cellular immune response parameters, dry matter intake (DMI) and average daily gain (ADG) in growing lambs after 0, 15, 30, 45 and 60 days of the experiment (n=5 lambs).

	Treatment				SEM	*P*	Day of Experiment					SEM	*P*		
	Control	EZ	SB	EZSB			D0	D15	D30	D45	D60		Linear	Quadratic	Cubic
Helper T lymphocytes	7.54	7.97	7.75	7.65	0.323	0.81	6.59^{b}	5.63^{c}	9.30^{a}	9.55^{a}	7.55^{b}	0.351	<0.01	0.09	<0.01
Cytotoxic T lymphocytes	40.23	38.47	40.47	41.01	0.934	0.28	37.8^{b}	43.7^{a}	33.4^{c}	41.0^{a}	44.3^{a}	1.04	0.89	0.42	<0.01
Granulocytes	38.59	38.18	37.79	38.23	0.596	0.82	38.36^{b}	43.26^{a}	31.41^{c}	38.45^{b}	39.51^{b}	0.568	<0.01	0.06	<0.01
Monocytes	9.87	9.8	9.2	9.48	0.366	0.55	5.86^{c}	6.16^{c}	9.53^{b}	13.24^{a}	13.14^{a}	0.388	<0.01	<0.01	0.18
DMI g/d	496.4	491.61	528.84	494.04	1.65	0.96	-	-	-	-	-	-	-	-	-
ADG	104.91	105.72	100.54	122.31	0.4357	0.7108	-	-	-	-	-	-	-	-	-

^{abc}Different superscripts following means within the same row and experimental factor indicate differences at P<0.05; SB, *S. babylonica* L. extract; EZ, exogenous enzyme; EZSB, *S. babylonica* L. extract + exogenous enzyme.

Table 3. Pearson's correlation coefficient (r) between average daily weight gain and cellular immune response parameters of growing lambs fed diet with addition of exogenous enzyme preparation (EZ) and *S. babylonica* L. (SB) extract as well as their mixture (EZSB) after 15, 30, 45, and 60 days of the experiment (n=5 lambs).

	Treatment								Day of experiment							
	Control		SB		EZ		EZSB		15		30		45		60	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Helper T lymphocytes	0.73	0.27	-0.12	0.88	0.77	0.23	0.58	0.42	-0.59	0.94	-0.87	0.13	-0.33	0.67	0.37	0.63
Cytotoxic T lymphocytes	-0.85	0.15	-0.60	0.40	-0.30	0.70	-0.97	0.03	0.45	0.55	-0.23	0.77	0.75	0.25	-0.21	0.79
Granulocytes	-0.88	0.12	-0.73	0.27	-0.38	0.62	-0.91	0.09	-0.64	0.37	0.30	0.70	0.39	0.61	0.04	0.96
Monocytes	0.12	0.86	-0.88	0.12	0.84	0.16	-0.06	0.94	-0.71	0.29	-0.24	0.76	0.58	0.42	-0.03	0.97

SB, *S. babylonica* L. extract; EZ, exogenous enzyme; EZSB, *S. babylonica* L. extract + exogenous enzyme.

was observed for granulocytes (r=-0.73, P=0.27) and monocytes (r=-0.88, P=0.12) as shown in Table 3. Helper T lymphocytes values were also not correlated to treatment EZSB (r=0.58, P=0.42), while they were negatively correlated to cytotoxic T lymphocytes (r=-0.97, P=0.03). Granulocytes tended to be negatively correlated (r= -0.91, P=0.09) to the same treatment.

Effect of the time of sampling on immunological parameters

The effect of time of sampling was different among treatments. Helper T lymphocytes values increased (P=0.01, linear and cubic effect), on day 30 and 45 compared with the day 15 when this value decreased. In the case of cytotoxic T lymphocytes, the values increased (P=0.01, cubic effect) on day 15, 45 and 60 and decreased on day 0 and 30. The values of granulocytes increased (P=0.01, linear and cubic effect), on day 15 compared to days 0, 30, 45 and 60 of the experiment. In accordance, monocytes values also increased (P=0.01, linear and quadratic effect) on day 45 and 60 and decreased on day 0 and 15 and 30 (Table 2). There were no correlations between some immunity parameters and ADG.

DISCUSSION

Effect of EZ on immunological parameters

There is no available literature on the impact of EZ on the immunological status in ruminants and in this study addition of EZ did not produce negative effects on immunological parameters. In a previous study, researchers did not observe adverse effects on animal health when they use 10 g of EZ per lamb/day (Salem *et al.*, 2011a).

However, enzymes are not routinely used in ruminant diets because it is generally assumed the enzyme proteins would be rapidly degraded by ruminal microbes (Beauchemin *et al.*, 1995; Beauchemin *et al.*, 2003). Addition of EZ to lamb diets did not resulted in correlations between ADG and the helper T lymphocytes and monocytes which suggests that when the animals are able to meet their nutrient requirements, they are capable of initiating immune response against antigens (Erickson *et al.*, 2000; Mitchell *et al.*, 2003; Enwonwu, 2006).

Effect of SB extract on immunological parameters

Extract of *S. babylonica* L. did not result in a detrimetal effect on immunological parameters in the present study, which was probably due to the low concentrations of secondary compounds (principally tannins and saponins) which were dosed daily to the lambs (Khalil and El-Adawy, 1994). Others studies have indicated negative effects of tannins and saponins on red and white blood cells counts resulting in anemia and immune deficiency (Adedapo *et al.*, 2005; Adedapo *et al.*, 2007; Mahgoub *et al.*, 2008). The absence of a negative effect on white blood cells in our study indicates that the SB extract is innocuous to this kind of cells. However, the same dose of SB extract used in other experiments showed anti-helmintic properties reducing intestinal worm

loads up to 40%, which represent an important effect for animal health, moreover, feed intake was not affected by extract addition as occurred in the present study (Salem *et al.*, 2011b).

We did not observe negative effects of ethanol present in the extract which in others studies has shown immunosuppressive effects by inhibition of enzymatic pathways (Hote *et al.*, 2008), therefore demonstrating that the ethanol content in the extract of *S. babylonica* L. is safe to lambs. Our results are not in accordance to those reported by Mahgoub *et al.* (2008) and Adedapo *et al.* (2007) who observed negative effects on white blood cells in sheep consuming diets rich in secondary compounds or extracts from different plants.

There were no correlations between ADG and the cytotoxic T lymphocytes, granulocytes and monocytes in SB lambs. These results suggested that when the animal presents increased immunological parameters, it cannot express its potential in production because the engulfing, presence or processing of the antigen may cause fever, emesis, diarrhea and anorexia (Grimble, 1998; Maggini *et al.*, 2007). This scenario promotes decrease in feed intake, ADG, milk production and other productive parameters (Maggini *et al.*, 2007).

There were negative ($r=-0.97$, $P=0.03$) correlations between ADG and cytotoxic T lymphocytes in EZSB group. This result suggests that when the animal present increased levels of such types of cells, it is processing an antigen which promotes a detrimental effect on ADG for the mechanism mentioned above. On the contrary, when the levels the cytotoxic T lymphocytes are low, the animal seems healthy which contribute to reach its maximal performance (Grimble, 1998; Maggini *et al.*, 2007).

Effect of the time of sampling on immunological parameters

The level of helper T lymphocytes in peripheral blood was linearly increased as the time of sampling advanced. These results indicate that treatments had negative effect (immunosuppressant) on helper T lymphocytes for the first fifteen days and then it had an immunostimulant effect on these cells, which remains for 30 days and then this effect began to diminish. Similar immunostimulant effects have been observed in fish treated with plant extracts and their products (Harikrishnan *et al.*, 2011). In ruminants, a period for adaptation while conducting nutrition experiments is very important, due to the fact that rumen micro-organisms require a minimum of 7 days to adapt to a diet. Thereafter, the micro-organisms begin to metabolize the dietary treatments (Van Soest, 1991). In this experiment, this time was evaluated in order to know the time it took to observe immunostimulant or immunosuppressant effects and also how long these effects remained. Mahgoub *et al.* (2008) evaluated the effect of secondary compounds at the beginning and at the end of the experiment, and they observed a decrement in the counts of this type of cells, nonetheless, this effect was weak at the end of the experiment.

The cytotoxic T lymphocytes augmented with time, and this increase was more

evident in the first 15 days. There was a more prolonged immunostimulant effect which suggests that the treatments had a positive effect on cytotoxic T lymphocytes. The effect on white blood cells is indirect although excessive ingestion of a wide variety of plants or their products (extracts) has been found to cause hypoproliferative or non-regenerative anemia. This cell disorder is characterized by reduced bone marrow production of all blood components in the absence of a primary disease process infiltrating the bone marrow or suppressing haematopoiesis. This process had a negative effect on neutrophils, lymphocytes, eosinophils, and monocytes (Adedapo *et al.*, 2007).

The time effect on granulocytes (neutrophils, eosinophils and basophils) was similar to cytotoxic T lymphocytes, with a stimulating effect on this cell type. These results may be due to the effect of extract saponins on cells and immune response (Erickson *et al.*, 2000; Harikrishnan *et al.*, 2011). Reports indicate that the function of neutrophils, eosinophils and basophils depends upon adequate consumption of macro and micronutrients; thus, malnutrition contributes to immune suppression (Mitchell *et al.*, 2003).

Regarding monocytes, our results suggest an immunostimulator effect because the animals them to 14 days have the capacity to metabolize and use any compounds presents in the diet. These results confirm that time is an important issue while feed additives are used and the period of adaptation to a particular diet as well (Van Soest, 1994).

CONCLUSIONS

The results suggest that exogenous enzyme and *S. babylonica* L. extract have immunostimulant effect the first 15 days, after these feed additives have immunosuppressive effects principally on cytotoxic T lymphocytes and granulocytes. The results indicate that EZ and SB as used in this experiment did not affect cellular immune response, thus, they might be used in lamb nutrition practices promoting growth performance without altering animal health. Both feed additives produced similar results supporting the use of *Salix babylonica* L extract as a suitable alternative when people cannot access to EZ.

The effect of the treatments throughout time ought to be considered in animal nutrition experiments. Studies evaluating the immune response might favour estimations of the effect of feed additives after the initial fifteen days.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from the IAEA, Vienna, Austria, Research Contract number MEX16307 within the D3.10.27 Coordinated Research Project. First author wish also to thank the National Council for Science and Technology (CONACYT, Mexico) for the scholarship for her doctoral program at the Universidad Autónoma del Estado de México.

REFERENCES

Adedapo, A.A., Abatan, M.O. and Olorunsogo, O.O. 2007. Effects of some plants of the spurge family on haematological and biochemical parameters in rats. *Veterinarski Arhiv*, 77: 29-38.

Adedapo, A.A., Adegbayibi, A.Y. and Emikpe, B.O. 2005. Some clinico-pathological changes associated with the aqueous extract of the leaves of *Phyllanthusamarus* in rats. *Phytotherapy Research*, 19: 971-976.

Ahmed, V.U., Perveen, S. and Bano, S. 1990. Saponin from the leaves of *Guaiacum officinale*. *Phytochemistry*, 29: 3287-3290.

AOAC. 1997. *Official Methods of Analysis*, 16th ed. Association of Official Analytical Chemists, Arlington, VA.

Arriola, K.G., Kim, S.C., Staples, C.R. and Adesogan, A.T. 2011. Effect of fibrolytic enzyme application to low- and high-concentrate diets on the performance of lactating dairy cattle. *Journal of Dairy Science,* 94: 832-841.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve eeed utilization by ruminants. *Journal of Animal Science,* 81: E37- E47.

Beauchemin, K.A., Rode, L.M. and Sewaltl, V.J.H. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. *Canadian Journal of Animal Science,* 75: 641- 644.

Bernfeld, P. 1955. Amylases, alpha and beta. *Methods in Enzymology*, 1: 149-158.

Calder, P.C. and Kew, S. 2002. The immune system: a target for functional foods. *British Journal of Nutrition*, 88: S165-S176.

Chandra, R.K. 2004. Impact of nutritional status and nutrient supplements on immune responses and incidence of infection in older individuals. *Ageing Research Review*, 3: 91-104.

Chung, Y.-H., Zhou, M., Holtshausen, L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M. and Beauchemin, K.A. 2012. A fibrolytic enzyme additive for lactating Holstein cow diets: ruminal fermentation, rumen microbial populations, and enteric methane emissions. *Journal of Dairy Science,* 95: 1419-1427.

Cowan, M.M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12: 564-582.

Enwonwu, C.O. 2006. Complex interactions between malnutrition, infection and immunity: relevance to HIV/AIDSinfection. *Nigeria Journal of Clinical and Biomedical Research*, 1: 6-14

Erickson, K.L., Medina, E.A. and Hubbard, N.E. 2000. Micronutrients and innate immunity. *Journal of Infectious Diseases*, 182: S5-10.

Gado, H.M., Salem, A.Z.M., Odongo, N.E. and Borhami, B.E. 2011. Influence of exogenous enzymes ensiled with orange pulp on digestion and growth performance in lambs. *Animal Feed Science and Technology,* 165: 131-136.

Grimble, R.F. 1998. Nutritional modulation of cytokine biology. *Nutrition,* 14: 634-640.

Harikrishnan, R., Balasundaram, C. and Heo, M.S. 2011.Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture*, 317: 1-15.

Hote, P.T., Sahoo, R., Jani, T.S., Ghare, S.S., Chen, T., Joshi-Barve, S., McClain, C.J. and Barve, S.S. 2008. Ethanol inhibits methionine adenosyltransferase II activity and S-adenosylmethionine biosynthesis and enhances caspase-3-dependent cell death in T lymphocytes: relevance to alcohol-induced immunosuppression. *Journal of Nutritional Biochemistry*, 19: 384-391.

Keith, M.E. and Jeejeebhoy, K.N. 1997. Immunonutrition. *Baillière's Clinical Endocrinology and Metabolism*, 11: 709-738.

Khalil, A.H. and El-Adawy, T.A. 1994. Isolation, identification and toxicity of saponin from different legumes. *Food Chemistry*, 50: 197-201.

Lin, Y., Means, G.E. and Feeney, R.E. 1969. The action of proteolytic enzymes on N,N-dimetyl proteins: basis for a microassay for proteolytic enzymes. *Journal of Biological Chemistry*, 244: 789-793.

Littell, R.C., Henry, P.R. and Ammerman, C.B. 1998. Statistical analysis of repeated measures data using SAS procedures. *Journal of Animal Science,* 76: 1216-1231.

Macey, M.G. 2007. Flow Cytometry: Principles and Aplications. Humana Press. New Jersey, USA.

Maggini, S., Wintergerst, E.S., Beveridge, S. and Hornig, D.H. 2007. Selected vitamins and trace elements support immune function by strengthening epithelial barriers and cellular and humoral immune responses. *British Journal of Nutrition*, 98: S29-S35.

Mahgoub, O., Kadim I, T., Tageldin, M.H., Al-Marzooqi, W.S., Khalaf, S.Q and Amnbu Ali, A. 2008. Clinical Profile of sheep fed non- conventional feeds containing phenols and condensed tannins. *Small Ruminant Research*, 78: 115- 122.

Mitchell, B.L., Ulrich, C.M. and McTiernan, A. 2003. Supplementation with vitamins or minerals and immune function: can the elderly benefit? *Nutrition Research*, 23: 1117-1139.

Nayak, S.K. 2010. Probiotics and immunity: A fish perspective. *Fish and Shellfish Immunology*, 29: 2-14.

NRC. 1985. *Nutrient Requirements of Domestic Animals*. National Research Council, Washington, DC, USA.

Owusu-Asiedu, A., Simmins, P.H., Brufau, J., Lizardo, R. and Péron, A. 2010. Effect of xylanase and β-glucanase on growth performance and nutrient digestibility in piglets fed wheat-barley-based diets. *Livestock Science*, 134: 76-78.

Robyt, J.F. and Whelan, W.J. 1972. Reducing valuemethods for maltodextrins: 1. Chain length dependence of alkaline 3,5-dinitrosalicylate and chain length independence of alkaline copper. *Analytical Biochemistry*, 45: 510-516.

SAGARPA. 1999. Norma Oficial Mexicana NOM-062-ZOO-1999, Especificacion estécnicasparala producción, cuidado y uso de los animales de laboratorio. DiarioOficial de la Federación.

Salem, A.Z.M., El-Adawy, M., Gado, H., Camacho, L.M., Ronquillo, M., Alsersy, H. and Borhami, B. 2011a. Effects of exogenous enzymes on nutrients digestibility and growth performance in sheep and goats. *Tropical and Subtropical Agroecosystems*, 14: 867- 874.

Salem, A.Z.M., Olivares, M., Lopez, S., Gonzalez-Ronquillo, M., R. Rojo, Camacho, L.M., Cerrillo, S.M.A. and Mejia, H.P. 2011b. Effect of natural extracts of *Salix babylonica* and *Leucaena leucocephala* on nutrient digestibility and growth performance of lambs. *Animal Feed Science and Technology,* 170: 27- 34.

Salem, A.Z.M., Robinson, P.H., López, S., Gohar, Y.M., Rojo, R. and Tinoco, J.L. 2010. Sensitivity of sheep intestinal lactic acid bacteria to secondary compounds extracted from *Acacia saligna* leaves. *Animal Feed Science and Technology,* 161: 85-93.

SAS. 2002. *SAS User's Guide: Statistics*. Ver 9.0. SAS Institute, Cary, N.C. USA, p. 956

Van Soest, P.J. 1991. *Nutritional Ecology of the Ruminant*, 2nd ed. Cornell University, USA.

Wintergerst, E.S., Maggini, S. and Hornig, D.H. 2007. Contribution of selected vitamins and trace elements to immune function. *Annals of Nutrition and Metabolism*, 51: 301-323.

Animal Nutrition and Feed Technology (2013) 13: 423-434

Influence of Jojoba Meal Treated with *Lactobacillus acidophilus* on Digestibility, Carcass Traits and Blood Metabolites in Growing Rabbits#

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ABSTRACT

El-Adawy, M.M., Abou-Zeid, A.E., Camacho, L.M., Salem, A.Z.M., Cerrillo-Soto, M.A. and El-Rayes, T. 2013. Influence of jojoba meal treated with L. acidophilus on digestibility, carcass traits and blood metabolites in growing rabbits. Animal Nutrition and Feed Technology, 13: 423-434.

The aim of the study was to evaluate the effect of substitution of soybean meal protein by *L. acidophilus* treated jojoba meal (JM) protein in rabbit's diets on digestibility of nutrients, N balance, caecotrophy, some blood metabolites and carcass traits. Thirty two 5-weeks-old rabbits were divided into four equal experimental groups, 8 rabbits of each in a complete random design. Soybean meal protein was partially replaced by treated JM protein at 0, 10, 20 and 30% to formulate the experimental diets. Feeding treated JM increased ($P<0.05$) of all nutrient digestibility except ether extract, accordingly nutritive values expressed as total digestible nutrients and digestible crude protein were improved ($P<0.01$) for diet contained 30% treated JM protein. N balance (g/day) was increased ($P<0.05$) in rabbits fed diet with 30% treated JM protein versus control. Incorporation of treated JM at 30% in the rabbit's diets increased ($P<0.05$) daily soft faces excretion, whereas crude protein the proportion of soft faces to total crude protein intake was higher ($P<0.05$). Plasma total protein, transaminases activity (GOT and GPT), glucose, creatinine and urea concentrations did not differ in JM supplementation diets versus control. Cholesterol and triglycerides concentrations decreased ($P<0.05$) with the substitution of JM protein in diets from 0 to 30%. Dressing percentage of the rabbit fed 30% treated JM protein was increased ($P<0.01$) by 5.1% compared to control diet. Supplementation of JM protein at 30% in rabbit's diets could increase nutrient digestibility, carcass traits quality and improve animal health.

Key words: Carcass traits, Digestibility, Jojoba meal, Lactobacillus, Rabbits.

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

Animal protein resources for human consumption can be partly met through rabbit's production. However, for intensified rabbit's production as poultry, it is necessary to develop low cost feed in developing countries. In Egypt, high feeding costs result from the shortage of protein sources used for animal feed. Therefore, there is a need to evaluate alternative protein sources to overcome such shortage problem. Jojoba (*Simmondsia chinensis*) is a shrub belonging to the Simmondsiaceae family which can be used as untraditional protein source in the animal diets. It is commonly grown in some countries such as, Argentina (2.0 hectares/year), Israel (1.1 hectares/year), and USA (1.1 hectares/year) and some Mediterranean and African lands. Around the world, 7930 hectares has been planted with jojoba shrub (Canoira *et al.*, 2006). In Egypt, the cultivation areas were concentrated in Ismailea region, New Valley, El-Sharkia and Assiut Governorates for increasing land reclamation (El-Rayes, 2010). High protein content of JM (from 20 to 30%) supports its potential as an ingredient for animal nutrition practices (Farag, 2007; El-Rayes, 2010). A limitation in the usage of JM as animal feedstuff might be due to its high content of simmondsin, simmondsin 2'-ferulate, and related cyano-methylenecyclohexyl glycosides (Van Boven *et al.*, 2000). Simmondsin is a toxic agent for broiler (Farag, 2007) and lambs (El-Kady *et al.*, 2008). The aim of the current study was to evaluate the effect of substitution of soybean meal protein by *L. acidophilus* treated JM protein in rabbit's diets on digestibility of nutrients, N balance, caecotrophy, some blood metabolites and carcass traits.

MATERIALS AND METHODS

Experimental procedures

Thirty two unsexed NZW rabbits, 5 weeks of age (650.5±5.5 g) were divided randomly into four equal experimental groups, 8 rabbits each. The experiment lasted for 12 weeks. Rabbits were housed in galvanized wire batteries of individual cages in a well ventilated building. Fresh water and diets were offered *ad libitum*. All rabbits were kept under the same managerial, hygienic and environmental conditions.

Four experimental diets were formulated to cover all the essential nutrient requirements for growing rabbits (De Blas and Mateos, 1998). The first group of rabbits was fed a basal diet containing soybean as a main source of protein. Soybean meal protein in the basal diet was partially replaced by treated jojoba meal (JM) protein with *L. acidophilus* at the level of 10, 20 and 30% to formulate the experimental diets. The composition and chemical analysis of the experimental diets are shown in Table 1.

The JM was treated with *L. acidophilus* according to the methodology of Verbiscar *et al.* (1981). *L. acidophilus* was enrichment in skimmed milk medium at 30°C for 8 days. After this period, JM was sprayed with this medium and rotating in a big bottle and incubated for 21 days at 26°C under anaerobic condition. The granular JM was ground using a hummer mill to pass a 4 mm screen. The meal was then dried

overnight in a forced air oven at 75°C, and stored in polyethylene bags until used for diet formulation.

Table 1. Composition (g/kg) and chemical analyses of the experimental diets

Ingredients	Level of JM protein (%)			
	0	10	20	30
Yellow corn	180.0	175.0	170.0	165.0
Wheat bran	165.0	161.0	158.0	155.0
Barley grain	130.0	135.0	140.0	145.0
Soybean meal[†]	150.0	135.0	120.0	105.0
Treated Jojoba meal[‡]	0.0	21.0	42.0	63.0
Berseem hay	330.0	328.0	325.0	322.0
Molasses	20.0	20.0	20.0	20.0
Limestone	10.0	10.0	10.0	10.0
Dicalsium Phosphate	6.0	6.0	6.0	6.0
Premix[§]	2.0	2.0	2.0	2.0
DL-Methionine	1.0	1.0	1.0	1.0
Commonsalt	5.0	5.0	5.0	5.0
Anti-toxicants	1.0	1.0	1.0	1.0
Chemical analyses				
Crude protein	166.6	165.8	164.2	161.9
Crude fibre	135.2	134.1	133.6	132.3
Ether extract	23.1	24.6	25.4	29.7
DL-Methionine	4.0	3.8	3.7	3.6
Lysine	10.3	9.7	9.2	8.9
Calcium	9.4	9.1	8.7	8.5
Total phosphorus	4.5	4.2	4.6	4.7
Digestible energy[¶], kcal/kg	2530	2558	2563	2582

[†]Soybean meal containing 400g CP/kg. [‡]Treated jojoba meal containing 285g CP/kg

[§]Each 3 kg of premix contained: Vitamin A 12000000 IU, vitamin D_3 2200000 IU, vitamin E 10000 mg, vitamin K3 2000 mg, vitamin B_1 1000 mg, vitamin B_2 4000 mg, vitamin B_6 1500 mg, vitamin B_{12} 10 mg, Pantothenic acid 10000 mg, Niacin 20000 mg, Biotin 50 mg, Folic acid 1000 mg, Coline chloride 500 g, Selenium 100 mg, Copper 10000 mg, Iron 30000 mg, Manganese 55000 mg, Zinc 50000 mg, Iodine 1000 mg and carrier $CaCO_3$ to 3000 g.

[¶]Calculated according to Fekete and Gippert (1985): Digestible energy (kcal/kg)=4253-32.8 (%CF)-144.4 (% ash).

Digestibility trial

A digestibility trial was performed using 16 male NZW rabbits at least three months of age and similar body weight to determine the nutrient digestibility coefficients and nutritive values of the four experimental diets. Four male rabbits in each group were housed individually in metabolic wire cages (50×50×40 cm) that allow collecting faeces and urine separately. Faecal and urinary samples were collected daily over 5 consecutive days according to the European reference method for rabbit digestion trials (Perez *et al.,* 1995). Sample of daily faeces (20%) of each rabbit were collected every day, dried at 60-70°C for 48 hrs, finally bulked, mixed, ground and kept for chemical analysis. Nutritive value in terms of total digestible nutrients (TDN) and the digestible crude protein (DCP) were calculated according to formula of Cheeke *et al.* (1982). For N balance, 10% of urine output was collected daily from each rabbit.

Sample analysis

Samples of offered feed and faeces individually taken weekly were mixed and milled through 1 mm screen and analysed for dry matter (DM, method 934.01), ash (method 942.05), nitrogen (N, method 954.01) and ether extract (EE, method 920.39) according to the AOAC (2000). Gross energy was determined by using an adiabatic bomb calorimeter. Digestible energy was calculated according to Fekete and Gippert (1985).

Semindosion was determined according to the method of Verbiscar *et al.* (1980) using the HPLC with a pump (Merck Hitachi, Germany). Samples (*i.e.,* 100g) of JM were ground to pass 1-mm, and extracted with acetone (2: 1, v/w) and refluxing for 4 h in a Soxhlet extractor. After acetone evaporation, simmondsin compounds (6.2 g of mixed toxicant) were obtained as a tan. These mixed toxicants (6.2 g) were dissolved in excess of acetone and washed through a short column containing merck silica gel 60 (230-400 mesh). The elution was concentrated to a smaller volume and acetone solution was allowed to evaporate slowly in a beaker and a product was rich in simmondsin crystallized. The simmondsin crystal was taken into 10 ml of methanol for HPLC analysis. Extraction of simmondsin was injected into a Rheodyne injector of the HPLC and samples were analysed on C_{18} silica gel column. The solvent was a mixture of methanol and water (20/80 v/v) and the flow rate was 1.0 ml/min. The column eluate was monitored at 217 nm with photodiode array detector. Calibration curve for simmondsin was obtained by plotting concentration ratio (simmondsin concentration/ concentration of internal standard) versus area ratio (area under simmondsin peak/area under internal standard peak).

Caecotrophy trial

At the end of the experiment (13 weeks), excretion of soft faeces and hard faeces were determined using four male rabbits per treatment. Plastic neck collars were used to prevent coprophagy. Soft and hard faeces of each rabbit were collected during 24 hours for three times according to the method described by Carabaño *et al.* (1988). The daily feed intake was recorded after deducting the scattered amounts. Sample of daily soft and hard faeces (about 20%) of each rabbit were taken for chemical analysis. The daily faecal samples collected were sprayed with 1% boric acid solution to prevent ammonia losses during drying. Faecal samples were dried at 70-80°C for 48 h. The dried faeces observed from each rabbit during the collection period were weighed, mixed, ground and kept for chemical analysis. Relative contribution of soft faeces to dry matter and crude protein intake were calculated according to Fraga *et al.* (1991) as follows:

Relative contribution of soft faeces to DM intake= (Soft faeces excretion, g DM/day)/ (Feed intake, g DM/day + Soft faeces excretion, g DM/day)×100.

Relative contribution of soft faeces to CP intake= (CP excreted in soft faeces, g/day)/ (CP ingested in feed, g/day + CP excreted in soft faeces, g/day)×100.

Carcass traits

Four rabbits from each treatment (2 males and 2 females) were randomly slaughtered at the end of the 14th week of age (Marketing age). Rabbits were weighed and slaughtered after fasting for 12 h (Lukefahr *et al.*, 1992). After slaughtering and complete bleeding (within 30 minutes), stomach, small intestine, caecum and large intestine weights were taken (full and empty) and carcass traits were evaluated according to Blasco *et al.* (1993). Hot carcass weight (HCW) was obtained 15-30 minutes after slaughter including liver, kidneys, head, lungs, esophagus, trachea, thymus and heart. Dressing percentage was estimated as HCW relative to pre-slaughter body weight. Cold carcass weight (CCW) was obtained after refrigerating the hot carcass between 0 and 4°C for 24 h. Giblets weight (liver, kidneys, heart and spleen), and carcass measurements were recorded.

Blood metabolites

Blood plasma was obtained by centrifugation of heparinized blood for 10 min at 3000 rpm and kept in eppendorf tubes until chemical analysis. Total protein in plasma was measured according to Tietz (1995). However, plasma albumin was measured according to Young (2001). Plasma globulin was calculated as the difference between plasma total protein and albumin. Glucose, cholesterol, triglyceride, GOT, GPT, urea and creatinine were determined using specific diagnostic kits produced by MDSS GmbH, Schiffgraben 41 (Germany) as recommended by Young (2001).

Statistical analysis

The experimental design was completely randomized. Eight rabbits per treatment were compared for digestibility and growth performance, while it was used only 4 rabbits for the evaluation of the carcass, and blood metabolites. Data were analysed using ''Mixed'' option of SAS (1996). The model included rabbits (random), jojoba protein supplementation level (fixed, 3 degrees of freedom (df)), and residual (rabbit within treatment). Levels of jojoba protein supplementation (treatments) were partitioned into linear contrasts. Significant differences were accepted when $P < 0.05$. Data were analysed according to SAS program (SAS, 1996). The application of the least of significance test for the differences among the different treatment means were done according to Duncan (1955). The following model was used:

$$Yij = \mu + Ti + eijk$$

Where, Yij=an observations; μ=Overall mean; Ti=Effect of using different levels of treated jojoba meal (i=0, 10, 20, and 30) and eijk=Residual (Random error).

RESULTS

Crude protein of treated JM increased by 19 points compared with untreated JM (Table 2), while other nutrients (ether extract, crude fiber and ash were not affected by treated JM with *L. acidophilus* bacteria, while gross energy content was also improved in treated JM.

Table 2. Nutrients and simmondsin concentrations (g/kg) and growth energy content (kcal/kg) of untreated and treated JM with *L. acidophilus*

	Untreated	Treated
Crude protein	266.2	285.2
Ether extract	113.7	111.2
Crude fiber	100.3	97.7
Ash	31.3	32.5
Simmondsin	36.0	1.6
Gross energy (kcal/kg)	5102	5114

Partial replacement of soybean meal protein by treated JM protein improved ($P<0.01$) all nutrient digestibility except ether extract (Table 3). Rabbits fed diet with treated JM protein at level 30% had the highest nutrient digestibility coefficient followed by those received the diets containing 20, 10 and 0%, respectively. Rabbits fed the 30% JM diet recorded the highest values of dry matter (DM), organic matter (OM), crude protein (CP) and crude fibre (CF) digestibility among the experimental diets, which were improved ($P<0.01$) by 12.0, 13.4, 11.0, 60.9%, respectively, *versus* control rabbits. Digestible CP of rabbits fed 20 and 30% JM diets increased ($P<0.01$) by 7.57 and 9.96%, respectively, compared to the control rabbits. Total digestible nutrient in rabbits fed diets with 20 and 30% JM increased ($P<0.01$) by 9.65 and 16.14%, respectively. Digestible energy (DE) of the experimental diets followed similar trend of the DCP and TDN, where the replacement of 10, 20 and 30% diet soybean meal protein by JM increased ($P<0.01$) DE by 1.1, 1.3 and 2.1% respectively. The partial replacement of soybean meal protein by treated JM protein at level of 30% in rabbit diets, increased ($P<0.01$) the digested-N, N-retained and N balance by 13.9, 18.8 and 3.9%, respectively, compared with control (Table 4).

Table 3. Effect of different levels of treated jojoba meal protein on nutrient digestibility and nutritive values of growing NZW rabbits

Items	Level of JM protein (%)				SEM†	P
	0	10	20	30		
Digestibility (%)						
Dry matter	703^{c}	714^{c}	752^{b}	787^{a}	23.7	**
Organic matter	722^{c}	727^{c}	779^{b}	819^{a}	11.3	**
Crude protein	709^{b}	714^{b}	781^{a}	787^{a}	9.8	**
Crude fibre	248^{c}	253^{c}	357^{b}	399^{a}	30.1	**
Ether extract	777	779	786	788	21.3	ns
Nutritive values						
TDN (g/kg)	632^{c}	64.6^{c}	69.3^{b}	734^{a}	22.7	**
DCP (g/kg)	118.1^{b}	118.4^{b}	128.2^{a}	127.4^{a}	2.41	**
Digestible energy (kcal/kg)	2530^{c}	2558^{b}	2563^{b}	2582^{a}	18.7	**

^{abcd}Means in the same row with different superscripts are significantly different ($P<0.05$); Linear at $P< 0.05$; **= $P< 0.01$; ns= Non significant

†SEM= Standard error of means.

Table 4. Effect of different levels of treated JM on N utilization of growing NZW rabbits (n=4)

	Level of JM protein (%)				SEM†	P
	0	10	20	30		
N intake (g/d)	3.33	3.44	3.40	3.45	0.43	ns
Faecal N (g/d)	1.02	1.03	1.04	0.83	0.38	ns
Urinary N (g/d)	1.02^{c}	1.28^{a}	1.02^{c}	1.10^{b}	0.49	*
N digested (g/d)	2.31^{b}	2.41^{b}	2.36^{b}	2.62^{a}	0.27	**
N retained (g/d)	1.29^{b}	1.13^{b}	1.34^{a}	1.52^{a}	0.42	**
N balance, % of N intake	38.73^{a}	32.84^{b}	39.41^{a}	44.05^{a}	1.12	**
% of N digested	55.84^{b}	46.88^{c}	56.77^{a}	58.02^{a}	1.47	*

^{abc}Means in the same row with different superscripts are significantly different (P<0.05);

Linear at P< 0.05; *= P<0.01; **= P<0.01; ns= Non significant

†SEM= Standard error of means.

Soft faeces CP was higher (P<0.01) in rabbits fed diets containing 20 or 30% treated JM protein by 11.3 and 18.6%, respectively, while, it decreased (P<0.01) in hard faeces by 11.5 and 12.7%, respectively (Table 5 and 6). CF content in soft faeces decreased (P<0.01) by 6.28 and 6.28%, respectively, in rabbits fed diets with 20 or 30% treated JM protein. However, in hard faeces, CF was decreased (P<0.01) by 6.34% in rabbits fed diets with 30% treated JM protein versus control rabbits.

No significant differences were observed in the carcass traits among treatments except for carcass and dressing weight, and relative liver of the pre-slaughter weight. Carcass and dressing weight increased (P<0.01) by 2.8 and 5.1%, respectively, in the rabbits fed 30% JM diets. In addition, relative liver of the pre-slaughter weight increased (12.9% - P< 0.05) in the rabbits fed diet with 30% JM (Table 7).

Table 5. Effect of different levels of treated JM on chemical composition of hard and soft faeces (g/kg) of growing NZW rabbits

	Level of jojoba meal protein (%)				SEM†	P
	0	10	20	30		
Soft faeces						
Dry matter	347.0	342.0	351.0	349.0	17.3	ns
Crude protein	264.0^{b}	271.0^{b}	297.0^{a}	313.0^{a}	14.7	*
Ether extract	12.8	13.3	13.9	14.7	4.3	ns
Crude fiber	177.6^{a}	176.0^{a}	166.3^{b}	167.1^{b}	7.8	**
Hard faeces						
Dry matter	482.5	497.1	493.8	475.0	16.3	ns
Crude protein	124.5^{a}	126.7^{a}	111.7^{b}	110.5^{b}	4.1	**
Ether extract	13.9	18.1	19.7	20.9	3.6	ns
Crude fiber	300.3^{a}	291.8^{a}	291.7^{a}	282.4^{b}	7.6	**

^{ab}Means in the same row with different superscripts are significantly different (P<05);

Linear at P<0.05; **= P<0.01; ns= Non significant

†SEM= Standard error of means.

Table 6. Effect of different levels of treated JM protein meal on soft faeces excretion of growing NZW rabbits

	Level of JM protein (%)				SEM†	P
	0	10	20	30		
Dry matter intake, g/d	127[c]	131[b]	137[a]	138[a]	2.83	***
Soft faeces excretion, g						
Dry matter/d	19.2[d]	20.6[c]	24.7[b]	26.2[a]	0.47	***
Dry matter/100 g	0.81[b]	0.83[b]	0.98[a]	1.03[a]	0.050	**
Chemical composition, g/kg						
Dry matter	347	342	351	349	7.3	ns
Crude protein	264[b]	271[b]	297[a]	313[a]	4.7	***
Relative contribution of soft faeces to						
Dry matter intake	13.13[b]	13.59[b]	15.28[a]	15.96[a]	0.31	**
Crude protein intake	19.33[c]	20.44[c]	24.61[b]	26.85[a]	0.25	**

[abcd]Means in the same row with different superscripts are significantly different (P<05);

Linear at P<0.05; **= P<0.01; ns= Non significant

†SEM= Standard error of means.

Table 7. Effect of different levels of treated JM on carcass traits (g/kg of live body weight) of growing NZW rabbits (n=4)

	Level of JM protein (%)				SEM†	P
	0	10	20	30		
Live body weight (g)	2046	2148	2120	2050	63.2	ns
Hot carcass weight, g	1108	1163	1150	1146	24.6	ns
Carcass (%)	54.23[b]	54.24[b]	54.41[b]	55.77[a]	1.08	**
Dressing	592.5[c]	602.8[bc]	608.9[b]	622.7[a]	20.40	**
Fur	147.1	146.9	148.6	150.7	5.70	ns
Total giblets weight	40.7	47.9	41.9	45.7	3.51	ns
Liver	30.2[b]	36.8[a]	34.4[a]	34.1[a]	1.61	*
Heart	2.8	3.1	3.0	3.1	0.90	ns
Kidneys	7.7	8.0	8.3	8.6	3.11	ns
Spleen	0.5	0.6	0.5	0.5	0.22	ns
Lungs	6.0	6.1	6.2	6.0	1.55	ns
Digestive tract (full)	199.2	197.7	198.8	203.7	8.91	ns
Digestive tract (empty)	.6.0	75.2	76.4	76.9	18.14	ns

[abcd]Means in the same row with different superscripts are significantly different (P<05);

Linear at P<0.05; **= P<0.01; ns= Non significant

†SEM= Standard error of means.

No significant differences were registered among treatments in some blood plasma parameter such as total protein, glucose, GPT, GOT, creatinine and urea (Table 8), whereas, plasma albumen decreased (P<0.05) by 8.2% in rabbits fed 30% JM diet compared to those fed control diet.

Table 8. Effect of different levels of treated JM on some blood metabolites of growing NZW rabbits

	Level of JM protein (%)				SEM†	P
	0	10	20	30		
No. of samples	4	4	4	4	--	--
Total protein, g/dl	5.8	6.1	5.7	5.9	0.43	ns
Albumin, g/dl	4.2[a]	4.4[a]	3.9[b]	3.8[b]	0.23	*
Globulin, g/dl	1.7[b]	1.7[b]	1.8[a]	2.1[a]	0.18	*
Glucose, mg/dl	128.7	127.9	128.2	127.4	0.89	ns
GPT, U/l	19.4	19.7	18.9	19.5	3.98	ns
GOT, U/l	50.1	50.4	49.7	49.5	7.11	ns
Cholesterol, mg/dl	91.2[a]	90.0[a]	81.2[b]	80.1[b]	9.18	**
Triglycerides, mg/dl	158.9[a]	158.8[a]	152.8[b]	152.3[b]	5.53	**
Creatinine, mg/dl	0.82	0.85	0.79	0.81	0.190	ns
Urea-N, mg/dl	20.6	20.8	20.1	20.4	2.89	ns

[ab]Means in the same row with different superscripts are significantly different (P<05);

Linear at P<0.05; **= P<0.01; ns= Non significant

†SEM= Standard error of means.

DISCUSSION

Chemical composition and detoxification of JM

The increase of CP in treated JM may be due to the influence of *L. acidophilus* bacteria, it converts the cyano-glycoside compounds such as (simmondsin and simmondsin -2'- ferulate) to microbial protein in their bodies. Studies indicated that *L. acidophilus* was grown on cyano-glycoside compounds as a sole carbon and nitrogen source in JM, producing some proteloytic enzymes for cyano-glycosides, and converted them to carbon chains and amides compounds (El-Shennawy, 2003). The bacteria consumed these compounds and used it in their bodies as microbial protein as previously mentioned by Abbott *et al.* (1999). Detoxification of JM has focused on the degrading of simmondsin by biological treatment with *L. acidophilus* bacteria as the principal toxic component in JM. Simmondsin contents in treated JM with *L. acidophilus* decreased by 96% as compared with untreated. This result may be due to the ability of *L. acidophilus* bacteria to produce some proteloytic enzymes for cyano-glycoside compounds and converted them to carbon chain and amides compounds. These resulting compounds were less toxic than simmondsin compounds (Abbott *et al.*, 1999).On the other hand, no differences in all nutrient contents (DM, CF, EE and ash) between raw and treated JM. Also, no significant differences in amino acids and fatty acids content between raw and treated JM. These results were nearly similar as described by earlier workers (Farag, 2007; Khalel *et al.*, 2008; Khayyal *et al.*, 2009).

Digestibility and N balance

The improvement of nutrient digestibility and nutritive value in JM rabbit's diets versus control may be attributed to some of the compounds produced from biological

treatments, which activate the digestibility or increase the caecum microbial activity (Khayyal *et al.,* 2009). The improvement in nutrient digestibility could be a result of better feed intake and nutritive value. These results are compatible with those observed by Khalel *et al*. (2008) and Khayyal *et al*. (2009) who pointed out that the nutrient digestibility coefficients were increased in rabbits fed 10% treated JM with *L. acidophilus* bacteria versus control rabbits. In this concern, Nelson *et al*. (1979) reported that fermentation of JM clearly improved its palatability, acceptability and digestibility coefficients in ruminants. These result are in accordance with those observed by Khayyal *et al*. (2009) who indicated that the nitrogen utilization of rabbits fed 10% treated JM with *L. acidophilus* bacteria were significantly increased as compared to control.

Results obtained from the chemical composition of soft and hard faeces (Tables 5 and 6) may indicate that dietary replacement of soybean protein meal by treated JM protein at levels of 20 and 30% activate the useful micro-organisms in caecum and colon, producing high crude protein content in soft faeces and improving the nutrients digestibility. Dietary treated JM protein levels effected ($P<0.01$) DM and CP contribution of soft faeces to total DM and CP intake, this is mainly due to higher DM and CP intake in the rabbits fed high level of treated JM (El-Sayaad *et al.*, 1998). The improvement of fiber digestibility coefficient leads to increase the rate of microbial protein synthesis in soft faces. The elevated microbial protein is the result of increased fiber utilization offering a suitable environment to bacterial growth in the caecum.

Carcass traits

Increments in carcass and dressing weights in rabbits fed 30% JM diets (table 7) may be due to improvements in body weight and daily gain. In general, the increase in body weight and daily weight gain in rabbits fed diets with 20 and 30% JM could be attributed to a favorable feed conversion ratio. The improvement in feed conversion may be due to the increase of protein and fiber digestibility coefficient as noticed in the current investigation. Supplementation of JM increased relative liver of the pre-slaughter weight, and these results are consistent with Khayyal *et al*. (2009) who reported that rabbits fed diets containing 10% JM with lactic acid bacteria increased carcass, dressing and liver weight as compared to those fed control diet. In comparison to poultry, Decuypere *et al*. (1996) reported that broiler breeder pullets fed diet containing 4% raw JM had increments in liver weight. On the other hand, Lisk and Brown (1985) found that live weight, carcass weight and dressing percentage of lambs fed diets containing 10% JM were not significantly affected.

Blood plasma metabolites

Decreased plasma albumin in JM rabbits may due to the higher CP digestibility observed in rabbits fed 30% JM diet. The same trend was noticed with raw JM by Khayyal *et al*. (2009). Plasma globulin increased ($P<0.05$) by 25.3% in rabbits fed diet

containing 30% JM compared to those fed control diet, this scenario might have be result of kidney dysfunction (Nephrosis). This result refers to the positive effect of JM diet on the immune response. However, plasma cholesterol and plasma triglyceride decreased ($P<0.05$) by 12.1 and 4.2%, respectively, in rabbits fed 30% JM diet versus control group probably due to the effect of biological treatment to reduce the concentration of saturated fatty acids and improving the unsaturated fatty acids in JM. These results are compatible with those obtained by Khayyal *et al.* (2009) who observed a significant decrease in both plasma cholesterol and triglycerides concentrations in rabbits fed diets containing 10% JM with *L. acidophilus* versus control. El-Kady *et al.* (2008) noticed that there were significant differences in the concentration of plasma triglycerides and cholesterol among groups of lambs fed different levels of JM. Where the group fed 30% raw JM increased ($P<0.01$) by 26.96 and 46.00%, respectively versus control. However, El-Shennawy (2003) found that there were no significant differences of plasma triglycerides and cholesterol concentrations among groups of rats fed untreated and treated JM with irradiation, heat, microwave and fermentation as compared to the control.

CONCLUSIONS

Treated JM by *L. acidophilus* bacteria could eliminate the harmful effect of its content of anti-nutritional factors and improve the effect on nutrient digestibility, caecotrophy, carcass traits and some blood plasma metabolites in rabbits. Using the JM at 30% in rabbit's diet could improve rabbit's health by reducing blood cholesterol and triglyceride concentrations and improve the immune response.

REFERENCES

Abbott, T.P., Holser, R.A., Plattner, B.J., Plattner, R.D. and Purcell, H.C. 1999. Pilotscale isolation of simmondsin and related jojoba constituents. *Industrial Crops and Production*, 10: 65-72.

AOAC. 2000. *Official Methods of Analysis,* 17th ed. Association of Official Analytical Chemists, Washington, DC.

Blasco, A., Ouhayoun, J. and Masoero, G. 1993. Harmonization of criteria and terminology in rabbit meat research. *World Rabbit Science*, 1: 1-10.

Canoira, L., Alcantara, R., Garcıa-Martınez, M.J. and Carrasco, J. 2006. Biodiesel from jojoba oil-wax: transesterification with methanol and properties as a fuel. *Biomass and Bioenergy*, 30: 76-81.

Carabaño, R., Fraga, M.J., Santoma, G. and De-Blas, J.C. 1988. Effect of diet on composition of cecal contents and on excretion and composition of soft and hard faeces of rabbits. *Journal of Animal Science,* 66: 901-10.

Cheeke, P.R., Patton, N. and Templton, G.S. 1982. *Rabbit Production*. 5th ed. The Interstate Printers and Publishers, Danville JII, USA.

De Blas, J.C. and Mateos, G.G. 1998. Feed formulation. In: *The Nutrition of the Rabbit*. (Eds. J.C. De Blas and J. Wiseman) Wallingford, CABI Publ., UK, pp. 241-254.

Decuypere, E., Van Wambeke, F., Vermaut, S., Buyse, J., Cokelaere, M., Flo, G. and De-Groote, G. 1996. Simmondsin: Physiological effects and working mechanisms in rats. In: *Proceedings on the 9^{h} International Conference on Jojoba and its uses*. (Eds. L.H. Princer and C. Rossi), pp. 137-141.

Duncan, B.D. 1955. Multiple Range and Multiple F. Test. *Biometrics*, 11: 1- 42.

El-Kady, I.R., Abou-Zeina, A.A.H., Omar, A.A.H., Salman, M.F., Shoukry, M.M. and Ahmed, M.S. 2008. Response of growing Ossimi lambs to diets containing different levels of defated jojoba meal. *American-Eurasian Journal of Agricultural and Environmental Sciences*, 4: 34- 43.

El-Rayes, T.K. 2010. *Effect of Jojoba Meal Supplementation as a Promising Feedstuff on Rabbit Performance*. MSc Thesis, Tanta University, Egypt.

El-Sayaad, G.A.E., Soliman, A.F. and Khayyal, A.A.M. 1998. Nutritional evaluation of sun dried rabbit manure in growing rabbit diets. *Egyptian Journal of Rabbit Science*, 8: 169-182.

El-Shennawy, H.M. 2003. *Nutritional and Toxicological Studies on Radiation Detoxification of New Protein Source*. PhD Thesis, Faculty of Science, Zagazig University, Sharkya, Egypt.

Farag, S.A. 2007. *Evaluation of Jojoba Meal as a Feedstuff in Broiler's Diet*. PhD Thesis, Tanta University, Egypt.

Fekete, S. and Gippert, T. 1986. Digestibility and nutritive value of Nineteen important feedstuffs for rabbits. *Journal of Applied Rabbit Research*, 8: 68-71.

Fraga, M.J., Perez De Ayala, P., Carabaño, R. and De Blas, J.C. 1991. Effect of type of fiber on the rate of passage and on the contribution of the soft faeces to nutrient intake of finishing rabbits. *Journal of Animal Science,* 69: 1566-1574.

Khalel, M.S., Hassan, A.A., Shwerab, A.M. and Khayyal, A.A. 2008. Feed evaluation of chemically or biologically treated jojoba meal. *Egyptian Journal of Nutrition and Feeds*, 3: 481-495.

Khayyal, A.A., Hassan, A.A., Shwerab, A.M., Khalel, M.S. and Salem, A.Z.M. 2009. Effect of feeding diets containing jojoba meal on growth performance of growing rabbits. *Egyptian Journal of Nutrition and Feeds,* 12: 475-489.

Lisk, D.J. and Brown, J.H. 1985. Feeding preferences; Oilmeals; Liveweight gain; Jojoba; Carcass quality; Finishing; Chemical composition. *In*: *Proceedings of the 6th International Conference on Jojoba and Its Uses*; ed. J. Wisniak and J. Zabicky, Beer-Sheva, Israel, pp. 343-353

Lukefahr, S.D., Van Vleck, L.D. and Roberts, J.D. 1992. Estimates of components of variance and covariance of carcass traits in rabbits using animal model. *Journal of Applied Rabbit Research,* 15: 259-273.

Nelson, E.A., Trei, J.E., Verbiscar, A.J. and Banigan, T.F. 1979. Palatability of various jojoba meal preparations with lambs. In: *Proceedings of the Western Section of the American Society of Animal Science*. July 28-August 1, 1979, University of Arizona, Tuscon, USA.

Perez, J.M., Lbas, F., Gidenne, T., Martens, L., Xicato, G., Perigi-Bini, R., Dallo-Zotte, A., Cossu, M.E., Carazzolo, A., Villamide, M.J., Carabaño, R., Fraga, M.J., Ramos, M.A., Cervera, C., Blas, E., Fernandez-Carmona, J., Falcão, E.C., Cmnha, M.L. and Bengala, F.J., 1995. European reference method for *in vivo* determination of diet digestibility. *World Rabbit Science*, 3: 41-43.

SAS. 1996. *SAS/STAT User's Guide; Statistics*, Ver. 6.04, 4th ed. SAS Institute, Inc., Carry, NC.

Tietz, N.W. 1995. Specimen Collection and Processing; Sources of Biological Variation. *Clinical Guide to Laboratory Tests*, 3rd ed. AACC, Washington, DC.

Van Boven, M., Busson, R., Cokelaere, M., Flo, G. and Decuypere, E. 2000. 4-Demethyl simmondsin from *Simmondsia chinenses*. *Industrial Crops and Products*, 12: 203-208.

Verbiscar, A.J., Banigan, T.F., Weber, C.W., Reid, B.L., Trei, J.E., Nelson, E.A., Raffauf, R.F. and Kosersky, D. 1980. Detoxification of jojoba meal. *Journal of Agricultural and Food Chemistry,* 3: 571-578.

Verbiscar, A.J., Banigan, T.F., Weber, C.W., Reid, B.L., Swingle, R.S., Trei, J.E. and Nelson, E.A. 1981. Detoxification of jojoba meal by Lactobacilli. *Journal of Agricultural and Food Chemistry*, 29: 296-302.

Young, D.S. 2001. *Effect of Disease on Clinical Laboratory Tests*, 4th ed. AACC Press, Washington, DC.

Animal Nutrition and Feed Technology (2013) 13: 435-439

Effect of Exogenous Fibrolytic Enzymes on the Carcass Characteristics and Performance of Grain-Finished Steers[#]

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ABSTRACT

Vargas, J.M., Mendoza, G.D., Rubio-Lozano, De la Salud, M. and Castrejón, F.A. 2013. Effect of exogenous fibrolytic enzymes on the carcass characteristics and performance of grain-finished steers. Animal Nutrition and Feed Technology, 13: 435-439.

The objective of this study was to determine the impact of different levels of a fibrolytic enzyme in a finishing diet on steer performance and carcass characteristics. The dietary enzyme levels were 0, 2, 4 and 6 ppm. Beef steers (n=7 per treatment) were fed a diet containing 88% concentrate and 12% forage (dry basis) for 75 days. Hot carcass yield improved (linear and quadratic, $P<0.05$) with enzyme treatment and the shear force tended to be reduced (linear, $P<0.10$) as enzyme concentration increased. No significant differences were detected in gain, feed conversion and loin characteristics. Thus, it is concluded that fibrolytic enzymes do not affect steer performance but improve carcass yield and tenderness.

Key words: Beef, Carcass, Enzymes, Meat quality, Performance, Steers.

INTRODUCTION

The use of exogenous enzymes in feedlot diets improves gain (Beauchemin *et al.*, 1999; Eivemark, 1997); however, evaluations have only reported on gain, feed efficiency and intake, regardless of probable changes in carcass and meat quality. In grain-based diets, the addition of enzymes increases digestion but the response depends on the dose of the enzyme and grain type (Beauchemin *et al.*, 2003). The addition of fibrolytic enzymes increase volatile fatty acid (VFA) concentrations and modify fermentation patterns (Ranilla *et al.*, 2008), but the response varies with the substrate

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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and enzyme used (Beauchemin *et al.*, 2003; Pinos-Rodriguez *et al.*, 2002). These changes could affect fat synthesis in the animal and carcass characteristics.

Feedlot cattle are commonly finished on high concentrate diets with a minimal levels of roughages, included to maintain healthy digestive tract function, however, the average feedlot diets has been reported to contain 26% NDF with a range of 13-41% (Busby *et al.*, 1999) with a low digestion, usually depressed up to 50% depending on the level of grain (Plata *et al.*, 1994) with differences among grains caused by the pH and rate of starch digestion (Martin *et al.*, 1999). It is possible that under limited conditions of cellulolytic bacteria growth and activity, the addition of exogenous enzymes may help to obtain digestible nutrients from the potentially digestible fraction that may represent 50% of the dietary NDF. Therefore, our objective was to determine if the dose of fibrolytic enzyme (in high grain diets) used in the Mexican beef industry could modify the performance and the carcass characteristics of commercial steers.

MATERIALS AND METHODS

Twenty-eight crossbred Zebu x Brown Swiss steers were used in this experiment. Animals were adapted to a high grain diet for 21 days. The enzyme product was Fibrozyme (Alltech Inc., Nicholasville, KY, USA) was dosed at 0, 2, 4 and 6 mg/kg of the diet. The enzyme is composed of fermentation extracts of *Aspergillus niger* and *Trichoderma viride* and activity was characterized by Ramirez *et al.* (2005) with 53.68 IU/g of cellulases and 222.16 IU/g xylanases. The finishing diet (dry basis) consisted on ground corn (60.07%), tallow (3.38%), soybean meal (12.95%), cane molasses (10.0%), chopped corn stover (12.0%) and mineral and vitamin premix (1.6%). The premix composition was: Ca 27%, P 3%, K 0.25%, Se 20 ppm, vitamin A 350,000 IU, vitamin D 150,000 IU, vitamin E 150 IU and lasalocid 2,000 ppm. The diet was fed in bunks at 08:00, 12:00 and 16:00 hrs daily. DM and protein content of feed were analysed by standard methods (AOAC, 1990); neutral detergent fiber (NDF), acid detergent fiber and lignin were determined using the procedures described by Van Soest *et al.* (1991) and gross energy was determined using an adiabatic calorimeter (Parr, USA). The chemical composition of the diet is shown in Table 1.

Table 1. Chemical composition of the basal diet

Constituent	Concentration
Dry matter, %	89.9
Crude protein, %	12.2
Neutral detergent fiber, %	17.6
Acid detergent fiber, %	6.20
Lignin, %	0.60
Gross energy, Mcal/kg	4.19

Cattle weights were recorded after the adaptation period and at the end of the fattening period (75 days). After slaughter, hot carcass weight was obtained. Muscle samples (approximately 50 g of the left side loin, *longissimus dorsi*) were collected and stored in a freezer (-20°C) until chemical analyses were performed. Warner Bratzler shear force (WBSF) and cooking loss were determined according to AMSA (1995). The steaks were broiled in open-hearth broilers to an internal temperature of 70°C, which was monitored with iron-constantan thermocouples (Omega Engineering Inc., Stamford, USA) and a recording portable thermometer. The steaks were turned over upon reaching 35°C, and allowed to cook until 70°C. After removal from the broiler, the steaks were allowed to cool to room temperature (20-25°C). Later, a minimum of eight 2.5 cm cores with a diameter of 1.27 cm were removed from each steak parallel to the muscle fiber and sheared once across the center of the core perpendicular to the muscle fiber in a Warner-Bratzler shearing device. The weight of each steak was measured prior to and after cooking, in order to calculate cooking loss. Objective color measurements were performed on two different sites of each steak, by means of a Minolta Chroma Meter CR-310 (Minolta, Osaka, Japan) after the steaks were allowed to bloom for 15 min at room temperature (20-25°C). The average lightness (L*), redness (a*) and yellowness (b*) of each sample were recorded. Data were analysed as a completely randomized design as described by Steel and Torrie (1980) and the linear and quadratic effects of enzyme level were tested.

RESULTS AND DISCUSSION

Daily intake, gain and feed conversion were not affected by the enzyme level (Table 2). Hot carcass yield increased as more enzyme was added (linear and quadratic $P < 0.05$; Table 2) and shear force tended to be reduced ($P < 0.10$).

Table 2. Effect of fibrolytic enzyme level on steer performance and carcass characteristics

	Enzyme level (ppm)				SEM
	0	2	4	6	
Dry matter intake, kg/d	10.7	10.1	10.9	11.7	1.10
ADG, kg	1.60	1.37	1.53	1.51	0.08
Feed conversion	6.68	7.37	7.12	7.74	0.22
Yield (hot carcass), %[†]	57.9	60.7	61.8	60.5	1.15
Loin characteristics[‡]					
L	40.9	40.9	40.6	38.5	1.12
A	22.7	23.0	21.7	21.5	0.60
B	10.4	9.6	11.0	8.0	0.80
Fat, cm	0.57	0.25	0.30	0.29	0.06
WBSF, kg/cm^2 [§]	5.27	5.31	4.99	4.67	0.43
Area, cm^2	63.2	61.2	68.3	65.1	5.21

[†]Linear and quadratic $P < 0.05$; [§]Linear $P < 0.10$

[‡]Loin characteristics: L, lightness; a, redness; b, yellowness; WBSF: Warner Bratzler shear force

Steer performance was not affected, as observed in other studies with different doses of commercial fibrolytic enzymes (Beauchemin *et al.*, 1997; Iwaasa *et al.*, 1997). However, Beauchemin *et al.* (1999) found ADG and feed conversion improved in grain-based diets, which was associated with increased feed digestion (Beauchemin *et al.*, 2003). One possible reason for the lack of a response is that the NDF content in the diet was very low. Schingoethe *et al.* (1999) found that reducing the concentrate and increasing the forage led to a good response to fibrolytic enzymes in dairy cattle rations. Another possible cause of non-response to the fibrozyme is that the dose used was very low compared to that reported in other experiments on steers, where doses have been greater than 10 grams per day (Gomez *et al.*, 2003; Gomez-Vazquez *et al.*, 2011).

Other feed additives, which do not alter digestion or metabolism, did not affect carcass yield and other characteristics (Meyer *et al.*, 2009). It is unclear why it was possible to modify the shear force with increasing dose; the ruminal fermentation pattern may have been altered as observed in other studies (Ranilla *et al.*, 2008); however, this was not determined in the present investigation.

CONCLUSION

It is concluded that fibrolytic enzymes do not affect steer performance but improve carcass yield and tenderness.

REFERENCES

AMSA. 1995. *Research Guidelines for Cookery, Sensory Evaluation and Instrumental Tenderness Measurements of Fresh Meat*. American Meat Science Association, Savoy, IL, USA.

AOAC. 1990. *Official Methods of Analysis*, 12th ed. Association of Official Analytical Chemists, Washington, DC, USA.

Beauchemin, K.A., Jones, S.D.M., Rode, L.M. and Sewalt, V.J.H. 1997. Effects of fibrolytic enzyme in corn or barley diets on performance and carcass characteristics of feedlot cattle. *Canadian Journal of Animal Science,* 77: 645-653.

Beauchemin, K.A., Rode, L.M. and Karren, D. 1999. Use of feed enzymes in feedlot finishing diets. Canadian *Journal of Animal Science,* 79: 243-246.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science,* 81: E37-E47.

Busby, D., Loy, D., Dewitt, D., Doran, B. and Irvin, R. 1999. An evaluation of effective fiber in beef feedlot finishing diets. Beef Research Report- Iowa State University. A.S. Leaflet R1634. www.extension.iastate.edu/…/beefreports/asl-1634.p.

Gómez, V.A., Pérez, P.J., Mendoza, M.G.D., Aranda, E. and Hernández, A. 2003. Fibrolytic enzymes improve performance in steers fed sugar cane and stargrass. *Livestock Production Science*, 82: 249-254.

Gomez-Vázquez, A., Mendoza, G.D., Aranda, E., Pérez, J., Hernández, A. and Pinos-Rodríguez, J.M. 2011. Influence of fibrolytic enzymes on growth performance and digestion in steers grazing stargrass and supplemented with fermented sugarcane. *Journal of Applied and Animal Research*, 39: 77-79.

Eivemark, S. 1997. Effect of fibrolytic enzymes in barley-based diets on performance of feedlot cattle and *in vitro* gas production. *Reproduction, Nutrition and Development*, 37: 83-84.

Martin, C., Philippeau, C. and Michalet-Doreau, B. 1999. Effect of wheat and corn variety on fiber digestion in beet steers fed high-grain diets. *Journal of Animal Science*, 77: 2269-2278.

Meyer, N.F., Erickson, G.E., Klopfenstein, T.J., Greenquist, M.A., Luebbe, M.K., Williams, P. and Engstrom, M.A. 2009. Effect of essential oils, tylosin, and monensin on finishing steer performance, carcass characteristics, liver abscesses, ruminal fermentation, and digestibility. *Journal of Animal Science,* 87: 2346-2354.

Pinos-Rodriguez, J.M., González, S., Mendoza, G.D., Barcena, R., Cobos, M.A., Hernández, A. and Ortega, M.E. 2002. Effects of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. *Journal of Animal Science,* 80: 3016-3020.

Plata, P.F., Mendoza, G.D., Bárcena, G.R. and González, M.S. 1994. Effect of a yeast culture (Saccharomyces cerevisiae) on neutral detergent fiber digestion in steers fed oat straw diets. *Animal Feed Science and Technology,* 49: 203-210.

Ramírez, C.L., Aranda, I.E., Mendoza, G.D., Palencia, L.L., Miranda, R.L.A. and Crosby, M. 2005. Caracterización de productos fibrolíticos comerciales utilizados en la alimentación de rumiantes. *Veterinaria México*, 31: 1-9.

Ranilla, M.J., Tejido, M.L., Giraldo, L.A., Tricarico, J.M. and Carro, M.D. 2008. Effects of an exogenous fibrolytic enzyme preparation on *in vitro* ruminal fermentation of three forages and their isolated cell walls. *Animal Feed Science and Technology,* 145: 109-121.

Schingoethe, D.J., Stegeman, G.A. and Treacher, R.J. 1999 Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. *Journal of Dairy Science,* 82: 996-1003.

Steel, R.G. and Torrie, J.H. 1980. *Principles and Procedures of Statistics*. McGraw-Hill, New York, USA.

Van Soest, P.J., Robertson, J. and Lewis, B. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3597.

Animal Nutrition and Feed Technology (2013) 13: 441-459

Mini Review: Basic Physiology and Factors Influencing Exogenous Enzymes Activity in the Porcine Gastrointestinal Tract#

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ABSTRACT

Strube, M.L., Meyer, A.S. and Boye, M. 2013. Minireview: Basic physiology and factors influencing exogenous enzymes activity in the porcine gastrointestinal tract. Animal Nutrition and Feed Technology, 13: 441-459.

The addition of exogenous enzymes to pig feed is used to enhance general nutrient availability and thus increase daily weight gain per feed unit. The enzymes used are mainly β-glucanase (EC 3.2.1.4) and xylanase (EC 3.2.1.8) and phytase (EC 3.1.3.8). Although *in vivo* data assessing feed enzyme activity during intestinal transit are few, it is known that the enzymes, being protein molecules, can be negatively affected by the gastrointestinal proteolytic enzymes and the low pH in the stomach ventricle. In this review, the pH-values, endogenous proteases and other factors native to the digestive tract of the adult pig and the piglet are discussed in relation to the stability of exogenous feed enzymes. Development of more consistent assessment methods which acknowledge such factors is warranted both *in vitro* and *in vivo*for proper evaluation and prediction of the efficiency of exogenous enzymes in the porcine gastrointestinal tract.

Key words: Feed Retention Time, Phytase, Xylanase, Glucanase, Stability, pH.

INTRODUCTION

With the advent of intensive animal husbandry, cost-effectiveness in feeding strategies has become an increasingly important issue. Cereals and oil-seed meals are widely used for feed, also in pig diets. The reliance of cereals and oil-seed meal as animal feed often result in only a selected few ingredients constituting the majority of the formulation, in turn increasing the potential detrimental effects of anti-nutritionalfeed factors in the feed such as viscosity inducing non-starch polysaccharides and phytate. The use of exogenous enzymes may attenuate these problems via catalyzing the degradation of the problematic compounds (Bedford and Schulze, 1998), and enzyme supplementation is now promoted in both adult pig as well as piglet feeds. The exogenous enzymes, typically microbially derived β-glucanase (EC 3.2.1.4), xylanase

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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(EC 3.2.1.8) and phytase (EC 3.1.3.8) activities, are usually added during feed formulation to act after ingestion, e.g. in the animal itself (Bedford, 2000). Motivations for enzyme use in animal feed thus include degradation of otherwise indigestible carbohydrates (Bedford, 1995) and decrease of viscosity which might otherwise negatively affect energy uptakeby forming unstirred layers on the surface of the epithelium (Rainbird *et al.*, 1984). Another incentive for enzyme addition to feed is to release micronutrients, such as the release of phosphate and other nutrients from phytic acid with phytase (Greiner and Konietzny, 2006; Selle *et al.*, 2000; Campbell and Bedford, 1992). Although research has shown that enzyme supplementation certainly may have an effect on nutrient utilization (Létourneau-Montminy *et al.*, 2012), the main purpose of this review is to evaluate to what extent the feed enzymes, being protein molecules, can 'survive' the primary digestion themselves and retain some activity in the gastrointestinal (GI) tract. Since enzymes are protein catalysts their activity is susceptible to variations in pH, and the enzyme proteins may be attacked by proteases acting inside the GI tract. Hence, for successful enzyme use it is important to assess the factors affecting enzyme activity and stability during passage.

Digestive physiology

In the monogastric animal, including pigs, digestion can be divided into four different stages: firstly, in the mouth, mastication provides mechanical degradation and addition of water as well as amylase (EC 3.2.1.1) (Arkhipovets, 1962). This step is not considered detrimental to feed enzymes. Next, the feed enters the stomach, where it encounters hydrochloric acid and pepsin (EC 3.4.23.1), both of which may compromise exogenous enzyme stability and, in turn, activity, via acid denaturation and proteolytic digestion, respectively. Afterwards the food bolus is slowly released into the small intestine, where the feed is neutralized by a pancreatic secretion containing bicarbonate and digestive enzymes including amylase, lipases and proteases (Corring, 1982). The main digestion and uptake of nutrients such as minerals, amino acids, sugars and fats takes place in the small intestine, and feed enzymes, being proteins, may thus be digested by the natural digestion processes. Eventually, the food bolus continues into the large intestine, where bacteria inactivate endogenous enzymes (Gibson *et al.*, 1989) and digest the remnants of the feed, mainly non-starch polysaccharides (NSP) and residual protein.

Retention time

As the different parts of the digestive tract present different challenges for preserving exogenous enzyme activity, the retention time in each segment is of utmost importance. The relevance of retention times is twofold: on the one hand, relatively long reaction times are required for the enzymes to accomplish a sufficient extent of substrate degradation; in particular, several hours may be required for degradation of non-starch polysaccharide fibres (Rasmussen and Meyer, 2010; Rasmussen *et al.*, 2012) and for full de-phophorylation of phytic acid (Bohn *et al.*, 2007). On the other hand, the

longer the enzyme is exposed to a hostile environment, the lower are the chances for it to retain its catalytic activity, due to acid denaturation and proteolytic digestion. In addition, most currently used exogenous enzymes have limited activity outside of a neutral or slightly acidic pH, and only low substrate conversion then occurs in the acidic stomach. The retention time in the stomach is thus a critical factor for retaining enzyme activity.

With cannulated pigs, retention times in the animals can be estimated by use of various indigestible compounds either fed or injected into the animal through cannules, allowing estimation f retention times in each segment independent of the previous one (Wilfart *et al.*, 2007; van Leeuwen and Jansman, 2007; Partanen *et al.*, 2007; Solà-Oriol *et al.*, 2010). One drawback of this approach is the fact that clearance from each segment follows exponential kinetics (Freire *et al.*, 2000; Snoeck *et al.*, 2004), e.g. the feed is not cleared from one segment before it reaches the next. Additionally, several different markers, both soluble and insoluble, may be used, and different algebraic approaches for estimation of the retention time may make it difficult to compare results, although an approximate assessment of the retention times is possible.

For the adult pig (see Table 1), reported retention times in the stomach range from 1 hour, independent of the fibre content of the diet (Wilfart *et al.*, 2007), to up to 13 hours on a high fibre, high water retaining diet as reported by van Leeuwen and Jansman (2007). A main reason for the differences is likely the feeding frequency, namely every fourth hour in the Wilfart *et al.* study (2007) compared to the twice a day regime in the Leeuwen and Jansman study (2007). The data thus suggest that a lower feeding frequency will cause retention in the stomach as the animal will eat larger portions on limited feeding frequency. In another study, approximately 50% of the feed was emptied from the stomach after 3 or 5 hours for a small and large meal, respectively (Gregory *et al.,* 2007). Johansen *et al.* (1996) used four different diets with different levels of fibre and reported gastric retention times of 3-5 hours with 3 x daily feeding, but no effect of diet type. The retention time in the small intestine is reported to be as short as 4 hours in the high frequency feeding study of Wilfart *et al.* (2007) and up to 21 hours with the high fibre, high water retaining diet of van Leeuwen and Jansman (2007). In other studies (Partanen *et al.*, 2007; Solà-Oriol *et al.*, 2010), where the stomach and small intestinal transit time were not differentiated, values of 6 to 10 hours have been reported, suggesting small intestine retention times of ~5 hours. Retention time in the colon is much longer than in the rest of the GI-tract, and ranges from 26 to 44 hours (Wilfart *et al.*, 2007; van Leeuwen and Jansman, 2007; Partanen *et al.*, 2007). Generally, shorter retention times appear to be associated with a higher content of NSP and more frequent feedings (Table 1).

In suckling piglets, retention times are much shorter due to their liquid diet, e.g. around 75% of a meal will typically leave the stomach after 1.5 hours as measured by gamma scintigraphy (Snoeck *et al.*, 2004). The literature reports varying retention times in the newly weaned pig. At weaning, where feed intake drops and the GI-tract is

compromised, the retention time increases substantially, as interpreted from findings indicating that 50% of the ingested feed is still present in the stomach at 8-16 hours, and is still not completely cleared after 50 hours (Snoeck *et al.*, 2004). Two-three weeks post-weaning, retention times along the GI-tract approach the ones in the adult pig, i.e. 3-7 hour mean gastric retention time (Snoeck *et al.*, 2004). In contrast it has been found in another study that the gastric retention times were less dependent of the age of the piglet post-weaning. Here, Wilson and Liebholz (1981) used very early weaning at 4 days of age, and observed mean gastric retention times of 1-2 hour at both 14 and 28 days of age. Retention times in the small intestine are difficult to accurately assess but small intestinal retention seem to be less than 7 hours in the piglet still suckling (Snoeck *et al.*, 2004) or around 2 hours in the piglet given milk (Wilson and Leibholz, 1981).Where pigs are offered solid feed, the retention rates are very variable, e.g. 4-28 hours (Snoeck *et al.*, 2004) to 2-4 hours (Wilson and Leibholz, 1981), although the

Table 1. Overview of reported retention times (given as hours) in the gastrointestinal tract of the adult cannulated pig. All values are not available as cannulation sites differ between studies. NSP: non-starch polysaccharides

Ref.	Method	Stomach (hours)	Small intestine (hours)	Colon (hours)	Total (hours)	Notes
Wilfart *et al.*, 2007	Cannulated growing pigs given 3 diets with 17-27% fibre every 4 hours (wheat bran).	1	4	26-44	31-50	Faster transit in small/large intestine with higher fibre content. High feeding frequency.
Van Leeuwen and Jansman, 2007	Cannulated pigs given 4 diets with different water holding capacity and NSP content fed 2/day.	4-13	5-21	45-73	67-85	Large variation, high NSP may increase transit speed in colon. Age and body weight may also increase transit speed.
Partanen *et al.*, 2007	Cannulated barrows given 3 diets with 20-25% fibre 2/day	7-10		30-34	35-44	Faster transit through colon with higher fibre content.
Solà-Oriol *et al.*, 2010	Cannulated pigs fed 4 diets of rice or oats and two feeding levels 2/day	6-10		-	-	Small increase in transit speed through small intestine with oats.
Gregory *et al.*, 2007	Cannulated pigs fed two feeding levels 2/day	3-5	-	-	-	Increased retention time with larger meals.
Johansen *et al.*, 1996	Cannulated pigs fed four different diets (wheat/oat) 2/day	3-5	-	-	-	No effect of diet type.

variance may stem from differences in measurement method and time from weaning. The differences here are likely to stem from the psychological stress of weaning and a decrease in feed intake. Total retention time is between 24 to 175 hours, depending on weaning status (Snoeck *et al.*, 2004; Freire *et al.*, 2000; Wilson and Leibholz, 1981).

In summary, the majority of the gastric content can be expected to be passed after 3-5 hours in the adult animal, but the retention time can be decreased by a higher meal frequency. Passage through the small intestine probably takes 5 hours, and the colon accounts for most of total the retention time, although this can be decreased with a higher content of fibre. In the young animal, the retention time is shorter for suckling pigs due to the liquid diet, but longer in the weaning and post-weaning piglet.

pH

The porcine stomach is acidic due to an influx of hydrochloric acid. Although the acidity of the stomach is often believed as being highly acidic, the actual pH is fairly variable, and may range from 1-5, due to a rapid buffering effect of immediate feeding behavior (Iraki *et al.*, 1997; Karamanolis *et al.*, 2008).

Adult pig

Gastric pH has been reported as being mostly above 4 during feeding on an *ad libitum* corn-based diet, but dropped to 2.5 at night and to 2.1 when feed was removed (Ange *et al.*, 2000). According to Potkins *et al.*, pH increased to 5.0 upon feeding with a large barley-based meal and then dropped below 3.0 after 7.5 hours, independent of bran or oat addition. If the barley meal was finely ground, however, it resulted in a significant drop to 2.5 after 7.5 hours (Potkins *et al.*, 2007). On a barley-based diet, pigs in a study by Bass and Thacker (1996) produced gastric digesta of pH 4.8, which dropped to 4.0 after 4 hours, although they sampled the digesta in the duodenum. Inborr *et al.* (1999) used a 40% wheat bran diet and observed a drop from pH 4.86 at feeding to pH 2.94 four hours later. Yi and Kornegay (1996) reported gastric pH in fed animals to be 3.69.

In the small intestine, pH-levels increase due to an influx of bicarbonate from the pancreas. It has recently been reported that the pH in the terminal ileum of barrows was 5.7-6.0, regardless of whether the diet was high in fibre and/or supplemented with organic acids or not (Partanen *et al.*, 2007). In contrast, the pH was found to range from 7.8 to 8.3 in the same segment in barrows on a high bran diet supplemented with enzymes (Inborr *et al.*, 1999).

Piglet

In the weaning piglet, a pH of 3.6 in the stomach has been reported on a liquid *ad libitum* diet (Hamilton and Roe, 1977). In piglets weaned 4 days old, duodenal, jejenual and ileal pH at 24 days was 5.9, 5.9 and 6.7, respectively, and a slight increase in pH with age was also observed (Wilson and Leibholz, 1981). Hamilton and Roe

(1977) observed a gradual increase from 6.0 to 6.7 from the duodenum to the ileum in the weanling piglet. Mathew *et al.* (1996) observed pH values of 6.40-7.45 in cannulated weaning piglets on a corn-based diet, where piglets weaned at 28 days rather than 21 days had lower final pH-values. Jejunal and ileal pH in weaning piglets on a corn/soy based diet was measured as between 7.0 to 7.4 in both segments (Franklin *et al.*, 2002). Piglets fed a corn/wheat/soy diet had a small intestinal pH between 5.9 and 6.7, where there was a slight alkalizing effect of a high protein diet in the ileum (Nyachoti, 2006). In summary, gastric pH in the fed animal is usually slightly below 5, and drops below 3 when the animal is fasted (overnight seem sufficient). The exact pH of the small intestine is fairly variable, but seems to be in the range from 5.7 to 8.3.

Effect on enzyme activity

It would seem that the stomach is a chemically more hostile environment than the small intestine, but it is difficult to distinguish a detrimental effect of low pH from the effect of pepsin (see later). According to Baas and Thacker (1996), who tested several commercial β-glucanases *in vitro*, only miniscule enzyme activity was evident below pH 4.5, and when enzymes were pre-incubated for 2 hours, they lost 40-80% activity at pH 2.5, 30-60% at pH 3.5 and 10-30% at pH 4.5. Cannulate samplings at the duodenum revealed high activity 15 min after feeding the same enzymes, but loss of 60-80% of enzyme activity after 120 min, although this could also be due to pepsin. None of the preparations resulted in any improvement of growth or improvements of digestibility when fed a barley based diet (Baas and Thacker, 1996). An analogous study, using the same enzyme preparations but testing xylanase activities, gave similar results, but showed even larger loss of enzyme activity (50-90%) after *in vitro* incubation at pH 2.5. There was, however, only limited loss when tested *in vivo* as per duodenal sampling after 120 min, and in this study, there was an improvement of daily weight gain in enzyme-supplemented pigs, although these pigs were fed a rye based diet (Thacker and Baas, 1996). Inborr *et al.* (1999) used a commercial Trichoderma preparation containing both xylanase and glucanase, and observed an almost 2-fold decrease within 2 hours and a 5 to 10 fold decrease by 4 hours of activity of both xylanase and β-glucanase activity in the stomach. The activity of xylanase and β-glucanase in the small intestine did not seem to decrease at all (Inborr *et al.*, 1999), providing strong evidence that these enzymes are more susceptible to the low pH of the stomach than to the conditions of the small intestine.

Phytases from microbial sources usually have pH-optima in the acidic range, and are reportedly stable below pH 3 (Greiner and Konietzny, 2006). Phytase activity in pigs fed a diet of soybean supplemented with phytase (Natuphos®) was 50%, 30% and 5% in the stomach, duodenum and ileum after slaughter, respectively (Yi and Kornegay, 1996). Jongbloed *et al.* (1992) added phytase (*Aspergillus niger* (var. *ficuum*) NRRL 3135) to the diets of cannulated pigs and in the duodenum, and observed 85% and 60% residual activity on a corn-based or tapiocha/hominy based diet, respectively. In the terminal ileum, no activity was detectable on either diet.

It seems that, as a broad generalization, glucanases and xylanases are more susceptible to low pH than phytases. Standardized methods to investigate the effect of pH isolated from digestive enzymes are warranted.

Endogenous digestive proteases

The porcine GI-tract produces several proteases, the most important of which are pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1). Since these actively digest proteins, they are a major obstacle for the use of exogenous enzymes, and proper estimation of proteolytic resistance of putative feed enzymes is warranted to predict *in vivo* performance. Pepsin is an aspartic protease produced in the stomach as pepsinogen, which is activated by low pH (Richter *et al.*, 1998). This activation happens within seconds below pH 2 but very slowly at pH 3-4, and also by an autolytic mechanism, both from within the individual protein and by other pepsin molecules (Al-Janabi *et al.*, 1972). The active enzyme has a pH-optimum of 2.0 and retains some activity up to pH 5.0, but is reversibly inactivated at pH 8.0 (Johnston *et al.*, 2007). The enzyme has highest specificity for peptide bonds having phenyl alanine, tyrosine, tryptophan and leucine in position P1 or P1' (Keil, 1992) (see Fig. 1 for notation). Trypsin and chymotryps in are the principal proteases of the small intestines, and both enzymes are produced in the pancreas. Trypsin is produced as trypsinogen, which is converted into the active trypsin by enteropeptidases in the duodenal wall, after which trypsin can activate additional trypsinogen. The pH optimum is 7-9 (Sipos and Merkel, 1970) and the preferred substrate is with arginine or lysine at P1 (Keil, 1992). Chymotrypsin is excreted by chymotrypsinogen, which is then activated proteolytically by trypsin. The pH optima of chymotrypsin is 7-9 and it preferentially cleaves at tryptophan, tyrosine and phenylalanine in position P1 (Keil, 1992). In practice, this translates to proteolytic activity of a very broad set of proteins. The difference in substrate preference of each of the proteolytic enzymes does, however, highlight the importance of testing putative feed enzymes with the proper combination of endogenous enzymes, e.g. pepsin followed by a combination of trypsin and chymotrypsin.

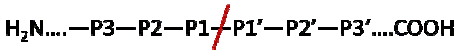

Fig. 1. Enzymatic cleavage scheme for proteases. Adapted from Schecter and Berger (1967).

Resistance of enzymes to endogenous proteolytic attack

In vitro testing of proteolytic resistance is usually done by incubation of exogenous feed enzymes for up to 2 hours with different ratios of endogenous proteases usually at a 0.001-0.1 ratio of endogenous protease (pepsin or trypsin) to exogenous feed enzyme (Pandee *et al.*, 2011; Rodriguez *et al.*, 1999; Rodriguez *et al.*, 2000). The current practice of using ratios of endogenous protease to feed enzyme does not take into account what the actual activity in the gastrointestinal tract is, and there are currently no clear data for structure-robustness of various enzymes against proteolytic attack other than by bioinformatical approaches (duVerle and Mamitsuka, 2012), which

in turn depend heavily on proper data sets. In addition, it is possible that both trypsin and chymotrypsin are required simultaneously for proper evaluation as these two enzymes may work synergistically considering their different bond attack targets (Gertler *et al.*, 1980).

The proteolytic resistance of different types of phytases differs, as evident from a study by Wyss *et al.*, where several fungal phytases were incubated with the proteolytic supernatant of an *Aspergillus niger* culture and showed markedly different degradation profiles (Wyss *et al.*, 1999). *In vitro*, a phytase from *Escherichia coli* named AppA phytase was initially activated by pepsin (0.1 mg/mL), after which activity fell to normal levels after 60 min. Trypsin (0.1 mg/mL) rapidly decreased activity to less than 50%, although a mutated enzyme was resistant (Zhu *et al.*, 2010). A phytase from *Neosartorya spinosa* BCC 41923 produced in *Pichia pastoris* retained most activity at low pepsin concentrations (pepsin/phytase ratio of 0.02), but only 25% activity was retained after 2 hours at a pepsin/enzyme ratio of 0.1 (Pandee *et al.*, 2011). For trypsin, a trypsin/phytase ratio of 0.001 retained an activity of 80%, but only 5% at a ratio of 0.01 (Pandee *et al.*, 2011). An *Aspergillus fumigates* phytase (r-Afp) was entirely resistant to pepsin at a ratio of up to 0.1, but lost 40% activity with trypsin at a pepsin/phytase ratio of 0.001 and full loss at 0.02 and above (Rodriguez *et al.*, 2000). In another study, *A. niger* r-PhyA and *E. coli* r-AppA was tested. The r-PhyA lost 20% activity at a 0.025 trypsin ratio, but 70% at 0.01 pepsin ratio. The r-AppA lost 80% at 0.025 trypsin, but gained 40% activity at 0.005 pepsin and above (Rodriguez *et al.*, 1999). Morgavi and Beauchemin (2001) was mainly concerned about the microbial inactivation of feed enzymes (*Trichoderma longibrachiatum*) in ruminants, but also tested the effect of pepsin at pH 3 and pancreatin at pH 7 (both from bovine sources, presumably). Pepsin, when used at 2mg/mL for 1 hour decreased activity of β-glucosidase and β-xylosidase by 10 and 15%, respectively, but did not affect activities of cellulase, cellulose 1,4-β-cellobiosidase or xylanase. Pancreatin may apparently increase the activity of cellulase, xylanase and cellulose 1,4-β-cellobiosidase substantially as a function of pancreatin concentration; in the case of cellulose 1,4-β-cellobiosidase, the activity increased linearly up to five-fold from 0 to 2.5 mg/mL of pancreatin addition. The loss of activity in a 60 min time-span was 0.1 to 0.55, 0.35 to 1, and 0.12 to 0.45%/min for cellulase, cellulose 1,4-β-cellobiosidase, and xylanase for the lowest and highest pancreatin concentrations, respectively. The authors speculate that limited proteolysis result in more efficient enzymes, a theory supported by at least two papers on cellulases (Eriksson and Pettersson, 1982; Chen and Grethlein, 1988). Despite the convincing data in one paper (Morgavi and Beauchemin, 2001), the proteolytic activation theory remains to be verified as it is in contrast to other reported results (see Table 2).

Table 2. Summary and estimated effect size of a selection of feed enzymes susceptibility to proteases found in the gastrointestinal tract. Values are residual activities after incubation with endogenous protease(s) or, if *in vivo*, measurements from cannulations. Only data with activity directly measured are included. Names or trademarks of enzymes are given when known. P:E: protease-to-enzyme ratio; Pancreatin: a mixture of pancreatic enzymes; *A: Aspergillus; E: Escherichia; T: Trichoderma*

Reference	Organism	Enzymes	Protease system	Activity after pepsin or stomach [%]	Activity after trypsin or small intestine [%]	Notes
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Yi and Kornegay, 1996	*A. ficuum*	Phytase	Cannulated pigs	30	5	*In vivo*
Zhang *et al.*, 2010	*A. ficuum*	Phytase	Pepsin and trypsin (1: 1 P:E)	100	100	
Rodriguez *et al.*, 2000	*A. Fumigates*	Phytase (r-Afp)	Pepsin (0.001-0.02) and trypsin (0.001-0.1 P:E), time not clear	100	64	
Zhang *et al.*, 2010	*A. niger*	Phytase	Pepsin and trypsin (1: 1 P:E)	70	10	
Jongbloed *et al.*, 1992	*A. niger (var. ficuum) NRRL 3135*	Phytase	Cannulated pigs	70	0	*In vivo*
Zhang *et al.*, 2010	*A. oryzae*	Phytase	Pepsin and trypsin (1: 1 P:E)	70	85	
Rodriguez *et al.*, 1999	*A.niger*	Phytase	Trypsin (0.005-0.025 P:E) and pepsin (0.002-0.01 P:E), 120min	80	30	
Luo *et al.*, 2009	*Bispora sp. MEY-1*	Phytase r-XYL11B	Pepsin and trypsin (0.1 P:E) 120min	100	25	
Luo *et al.*, 2010	*Bispora sp. MEY-1 CGMCC 2500*	β-1,3-1,4-glucanase (Bg17A)	Pepsin and trypsin, not clear how much, 60min	100	100	
Zhu *et al.*, 2010	*E. coli*	Phytase AppA	Pepsin (0.1 mg/mL), Trypsin (0.1 mg/mL), 60 min	110	50	
Rodriguez *et al.*, 1999	*E. coli*	Phytase	Trypsin (0.005-0.025 P:E) and pepsin (0.002-0.01 P:E), 120min	140	20	

Table 2. Contd.....

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Zhang *et al.*, 2010	*E.coli*	Phytase	Pepsin and trypsin (1: 1 P:E)	120	80	
Pandee *et al.*, 2011	*N. spinosa*	Phytase	Pepsin and trypsin (0.02-0.1 P:E), 120 min	0	20	
Peilong *et al.*, 2007	*Paenibacillus sp. F-40*	β-1,4-glucanase	Pepsin and trypsin, not clear how much, 60min	60	60	
Morgavi and Beauchemin, 2001	*T. longibrachiatum*	Cellulase	Pancreatin (2.5-0.075 mg/mL), pepsin (0.063-2 mg/mL), 60min	100	600	20 min lag-phase, then peak and slight decrease
Morgavi and Beauchemin, 2001	*T. longibrachiatum*	Cellulose 1, 4-β-cellobiosidase	Pancreatin (2.5-0.075 mg/mL), pepsin (0.063-2 mg/mL), 60 min	100	600	Slight dip after 40 min
Morgavi and Beauchemin, 2001	*T. longibrachiatum*	Xylanase	Pancreatin (2.5-0.075 mg/mL), pepsin (0.063-2 mg/mL), 60min	90	200	-
Morgavi and Beauchemin, 2001	*T. longibrachiatum*	β-glucosidase	Pancreatin (2.5-0.075 mg/mL), pepsin (0.063-2 mg/mL), 60min	90	55	
Cai *et al.*, 2011	*Penicillium pinophilum C1*	Xylanase XYN10C1	Pepsin and trypsin, concentration not clear, 120min	120	120	
Zhao *et al.*, 2012	*Phialophora sp. G5*	β-1,4-glucanase (GelG5)	Pepsin and trypsin (0.1 P:E) 60 min	90	90	
Roy *et al.*, 2012	*Shigella sp. CD2*	Phytase	Pepsin and trypsin (0.1mg/mL)	80	80	
Morgavi and Beauchemin, 2001	*T. longibrachiatum*	Xylanase	Pancreatin (2.5-0.075 mg/mL), pepsin (0.063-2 mg/mL), 60 min	90	500	
Inborr *et al.*, 1999	*T. longibrachiatum*	ß- (l,3) (l,4)-glucanase	Cannulated pigs sampled at stomach and cecum	15	115	*In vivo*, sampling in cecum may reflect native bacteria
Inborr *et al.*, 1999	*T. Viride*	ß- (l,4)-xylanase;	Cannulated pigs sampled at stomach and cecum	10	140	*In vivo*, sampling in cecum may reflect native bacteria

In vivo, it is difficult to distinguish between the effect of acid and protease, but phytase apparently has activity in the gastrointestinal tract: microbial phytase (Natuphos®, *A. niger*) added to a wheat-based diet resulted in twice the ileal phosphate absorption (34.3% vs. 17.6%) in cannulated pigs (Rapp *et al.*, 2001). Phytase (Danisco®, presumably Phyzyme® *E.coli*) addition to barley/pea-based diet in a 17-day experiment resulted in an increase in phosphate retention (40% vs. 57%) and a slight increase in nitrogen retention (Oryschak *et al.*, 2002). In cannulated pigs fed a maize/soy-based diet, disappearance of total phosphate was twice as high with added phytase (Natuphos®, *A. niger*) (30.5% vs. 59.9%) (Kemme *et al.*, 2006).Inborr *et al.* (1999) used a commercial *Trichoderma* preparation containing both xylanase and glucanase and observed no decrease in enzyme activity in the small intestine in spite of a marked loss in the stomach, and in a separate study the phytase activity in pigs fed a diet of soybean supplemented with phytase, activity decreased to 50-30% when sampled in the stomach. This activity was decreased to 5% or less in the terminal ileum (Yi and Kornegay, 1996). Jongbloed *et al.* (1992) saw a total loss of phytase activity (*A. niger (*var. *ficuum)* NRRL 3135) in the terminal ileum, even though 60-85% activity remained in the duodenum.

In vivo, it also appears that phytase (*A. niger)* supplementation in pigs decrease the activity of both trypsin and chymotrypsin, although it did not change apparent ileal digestibility of amino acids (Morales *et al.*, 2012).

Novel feed enzymes do seem to be very resistant to proteolysis by pepsin and trypsin in *in vitro* trials with residual activities of >90% (Zhao *et al.*, 2012), ~100% (Luo *et al.*, 2010) and 60% (Peilong *et al.*, 2007) for glucanases, ~100% (Zhang *et al.*, 2010) and 80% (Roy *et al.*, 2012) for phytases and 70-100% (Luo *et al.*, 2009) and >100% (Cai *et al.*, 2011)for xylanases. Based on the available data (Table 2), it would seem that enzymes used in animal feed are generally more susceptible to trypsin than pepsin. However, the measured extent of resistance *in vitro* obviously depends on the test methods, which are presently not standardized. The reported results obtained *in vitro* may moreover not necessarily reflect the *in vivo* situation correctly, in particular the exposure time may be too low *in vitro*, considering the extensive retention times prevailing in the genuine porcine GI tract (Table 1).

In general, it is not straight forward to properly estimate the susceptibility to proteases due to the inconsistencies of *in vitro* testing methodologies, e.g. using one or multiple proteases in different concentrations, and this is further complicated by the variety of enzyme sources from different organisms. Moreover, the relatively short exposure times generally used *in vitro* may be too short to properly reflect the *in vivo* exposure.

Bile salts

Bile acids are surfactants produced in the gallbladder, required for proper digestion of fats as they stabilize microsized fat droplets that are then more efficiently digested by lipases. Whether bile interfere with enzyme activity seemingly depend on the enzyme, as trypsin and chymotrypsin are entirely unaffected (Robic *et al.*, 2011) or facilitated (Gass *et al.*, 2007),whereas bacterial prolyl endopeptidases loose 2/3 of their activity in even sub-physiological concentrations of cholic acid, without the presence of trypsin or chymotrypsin (Robic *et al.*, 2011). ATP-ase activity was diminished by bile salts (Scharschmidt *et al.*, 1981). No data are available on how bile may affect the activity of feed enzymes, but enzyme supplementation has been suggested to increase the efficiency of the bile salts themselves (Campbell and Bedford, 1992). Fat digestion is more markedly improved by NSP-ases than digestion of other nutrients, which may be due to the decrease of viscosity of digesta, allowing for better micelle formation (Bedford and Schulze, 1998).

Water content and ionic strength

Enzymes are often dependent on ionic strength, inasmuch as they often exhibit limited activity at low salt concentrations, a definite peak at a certain concentration and then a decrease at higher concentrations. This, as an example, occurs around 0.1M NaCl for a fungal pectinase (Dahodwala *et al.*, 1974) and for a glucanase at 0.2mM NaCl (Sena *et al.*, 2011). Naturally, halophilic enzymes do not function optimally until NaCl concentration reaches 5-15% (Wainø and Ingvorsen, 2003).

Water content in the GI of piglets decreases 2.5 fold in the stomach, but increases in the duodenum to around 50% more than in the original meal. There is then no or a small net uptake of water until the distal jejunum, after which uptake increases markedly and is very high throughout the colon (Hamilton and Roe, 1977). In the same study, Na^+ was 24 mM and 80-100mM, K^+ was 25 and 10-38mM and Cl^- 112 and 26-93 mM in the stomach and small intestine, respectively, with an observable decrease of K^+ and Cl^- through the length of the small intestine (Hamilton and Roe, 1977). For the stomach and the small intestine, respectively, in adult animals, the following total concentrations were observed: 52 and 140 mM Na^+, 21 and 30mM K^+, 5-10 and 10-60 mM Ca^{++}, 5 and 5-20mM Mg^{++}, 0.1 and 0.2-0.6 mM Zn^{++}, 1 and 1-3 Fe^{++}, 0.02-0.04 and 0.04-0.1 mM Cu^{++}, evidently dependent on diet (Dintzis *et al.*, 1995). Measurement of divalent cations depends on the method as they are usually bound by phytate, and are usually not soluble and hence less detectable in the liquid phase on a high phytate diet (Woyengo *et al.*, 2010).

The concentrations of ions in the GI-tract of pigs does not appear to be an issue for enzyme stability, but rather, the ionic conditions may be more favorable in the small intestine than in the stomach. In addition, seeing as the lower water content in the stomach results in higher viscosity, which in turn results in lower enzyme activity (Uribe and Sampedro, 2003), the small intestine would be a more active site of action.

Other effects

The redox potential has not been extensively measured in the pig. A German study seems to show a decrease in redox potential to around -300mV in stomach and large intestine, but only a slight decrease in the small intestine from birth to 3 days in piglets, and apparently the same situation holds true for the adult animal (Schulze and Jacob, 1981). The reduction potential of DTT is -330mV (Cleland, 1964), and is routinely used to reduce disulfide bridges in biochemistry. If the reductive potential is sufficient to disrupt proteins in the stomach, this may be an overlooked factor in terms of stability, apart from pH and proteolysis.

Although particle size is an important factor for digestibility and may possibly increase enzyme effectiveness (Mavromichalis *et al.*, 2000; Oryschak *et al.*, 2002; Yáñez *et al.*, 2011), likely by increasing the surface area of the substrate, it does not appear that particle size has any direct effect on enzyme stability. Regarding the enzyme action, it is known that at least the phytase from wheat acts with significantly less efficiency, having both a lower K_M and a lower V_{max} on its naturally occurring substrate, this being phytate residing in globoids, in contrast to pure phytic acid (Bohn *et al.*, 2007). The latter appears to be widely used as substrate for assessing phytase activity.

Enzyme preparations with added exogenous proteases have been used in weaned pigs (Omogbenigun, Nyachoti, and Slominski, 2004) and in growing pigs (Olukosi *et al.*, 2007; Yin *et al.*, 2001) with favorable results, suggesting that these proteases do not digest other exogenous enzymes.

Test reactors

Test reactors are rarely used for testing animal feed enzymes, although testing is quite standardized for pharmacological dissolution profiles (Gray *et al.*, 2009; Wong *et al.*, 1997). Usually, the methodology used for testing gastric resistance includes 2 hours of pH 1.2 HCl (The United States Pharmacopeial Convention, 2007), which, on light of the observed pH in pigs, may be too severe (see earlier paragraph).Often, enzymes are simply incubated with various concentrations of either pepsin or trypsin for a defined period of time (Zhao *et al.*, 2012; Luo *et al.*, 2010; Zhang *et al.*, 2010; Wyss *et al.*, 1999). Apart from the lack of consensus in testing conditions, an issue is on how the successive conditions of the GI-tract may have interactive effects on the enzymes, e.g. slight denaturation by low pH and/or pepsin may prime proteins for further digestion by trypsin and chymotrypsin. When testing bacteria or drug systems for digestion resistance, systems such as the SHIME-reactor (Molly *et al.*, 1994) or more simple bioreactors with or without enzymes are often utilized (Siew *et al.*, 2000; Sumeri *et al.*, 2008; Ritter *et al.*, 2009). A fairly simple reactor, such as the ones proposed by Sumeri *et al.* (2008), with added enzymes at the appropriate times would be useful. This could be 3 hours at pH 3 with pepsin, followed by 5 hours at pH 6.5 with added trypsin and chymotrypsin (pancreatin could be a valid option). The exact concentrations of enzyme

is not clear, but according to Fang, trypsin was 43.92 U/mL (1 U=1 μmol TAME per min at 25°) and chymotrypsin was 6.89 U/mL (1 U=1 μmol BTEE per min at 25°) (Fang *et al.*, 2012).Morales *et al.* found 20-60U/mL trypsin and 4-10U/mL chymotrypsin (Morales *et al.*, 2012). Others have used different substrates for testing (Hedemann and Jensen, 2004; Wang *et al.*, 2008) or made 24 hour total estimate (Low, 1982), making comparisons difficult. Concentration of enzyme should be chosen to mimic reported values in the literature.

CONCLUSION

The following key physiological factors should be considered in relation to exogenous enzyme activity in the porcine gastrointestinal tract: 1) With a high feeding frequency, retention times in the stomach are as low as 1 hour but with lower frequencies, the gastric retention time is typically between 3-5 hours. 2) The pH in the stomach is between 3 and 5, depending on feeding levels, which is substantially higher than pH 1.2 typically used in model systems for assessing feed enzyme stability. 3) Enzymes are also strongly affected by proteolytic activities in the stomach as well as in the small intestine, apparently mostly to trypsin and other small intestinal enzymes. In conclusion, development of better test methods is needed to assess and predict the stability and activity of exogenous feed enzymes in the porcine gastrointestinal tract. Since retention time, endogenous proteases and pH are inherent properties of animal physiology, novel ways to protect enzymes warrant attention. Although addition of enzymes to porcine feed is already used, innovative strategies for enzyme protection and targeted delivery may provide for even better effects.

REFERENCES

Al-Janabi, J., Hartsuck, J.A. and Tang, J. 1972. Kinetics and mechanism of pepsinogen activation. *Journal of Biological Chemistry,* 247: 4628-4632.

Ange, K.D., Eisemann, J.H., Argenzio, R.A., Almond, G.W. and Blikslager, A.T. 2000. Effects of feed physical form and buffering solutes on water disappearance and proximal stomach pH in swine. *Journal of Animal Science,* 28: 2344-2352.

Arkhipovets, A. 1962. The amylolytic activity of pigs' saliva and its role in the digestive process. *Vestnik Sel'skokhoz. Nauki* 7: 86-88.

Bedford, M. 1995. Mechanism of action and potential environmental benefits from the use of feed enzymes. *Animal Feed Science and Technology,* 53: 145-155.

Bedford, M. 2000. Exogenous enzymes in monogastric nutrition—their current value and future benefits. *Animal Feed Science and Technology,* 86: 1-13.

Bedford, M. and Schulze, H. 1998. Exogenous enzymes for pigs and poultry. *Nutrition Research Reviews,* 11: 91-114.

Bohn, L., Josefsen, L., Meyer, A.S. and Rasmussen, S.K. 2007. Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. *Journal of Agricultural and Food Chemistry,* 55: 7547-7552.

Baas, T. and Thacker, P. 1996. Impact of gastric pH on dietary enzyme activity and survivability in swine fed beta-glucanase supplemented diets. *Canadian Journal of Animal Science,* 76: 245-252.

Cai, H., Shi, P., Bai, Y., Huang, H., Yuan, T., Yang, P., Luo, H., Meng, K. and Yao, B. 2011. A novel thermoacidophilic family 10 xylanase from *Penicillium pinophilum* C1. *Process Biochemistry,* 46: 2341-2346.

Campbell, G. and Bedford, M. 1992. Enzyme applications for monogastic feeds: a review. *Canadian Journal of Animal Science,* 466: 449-466.

Chen, H. and Grethlein, H. 1988. Effect of cellulase size recuction on activity and accessibility. *Biotechnology Letters,* 10: 913-918.

Cleland, W.W. 1964. Dithiothreitol, a new protective reagent for SH groups. *Biochemistry* 3: 480-482.

Corring, T. 1982. Enzyme digestion in the proximal digestion of the pig: a review. *Livestock Production Science,* 9: 581-590.

Dahodwala, S., Humphrey, A. and Weibel, M. 1974. Pectic enzymes: individual and concerted kinetic behavior of pectinesterase and pectinase. *Journal of Food Science,* 39: 1920-1926.

Dintzis, F.R., Laszlo, J.A, Nelsen, T.C., Baker, F.L. and Calvert, C.C. 1995. Free and total ion concentrations in pig digesta. *Journal of Animal Science,* 73: 1138-1146.

duVerle, D.A. and Mamitsuka, H. 2012. A review of statistical methods for prediction of proteolytic cleavage. *Briefings in Bioinformatics*, 13: 337-349.

Eriksson, K.E. and Pettersson, B. 1982. Purification and partial characterization of two acidic proteases from the white-rot fungus *Sporotrichum pulverulentum*. *European Journal of Biochemistry,* 124: 635-642.

Fang, L., Zhang, H., Zhao, F., Li, J.W. and Li, D. 2012. Determination of digestive enzyme activity in the digesta from the small intestinal of growing pigs and the development of *in vitro* evaluation system for feed bioavailability using artificial small intestinal juice. *Journal of Animal and Veterinary Advances,* 11: 1819-1826.

Franklin, M., Mathew, A., Vickers, J. and Clift, R. 2002. Characterization of microbial populations and volatile fatty acid concentrations in the jejunum, ileum, and cecum of pigs weaned at 17 vs. 24 days of age. *Journal of Animal Science,* 80: 2904-2910.

Freire, J., Guerreiro, A., Cunha, L. and Aumaitre, A. 2000. Effect of dietary fibre source on total tract digestibility, caecum volatile fatty acids and digestive transit time in the weaned piglet. *Animal Feed Science and Technology,* 87: 71-83.

Gass, J., Vora, H., Hofmann, A.F., Gray, G.M. and Khosla, C. 2007. Enhancement of dietary protein digestion by conjugated bile acids. *Gastroenterology* 133: 16-23.

Gertler, A., Abrahami, B. and Bondi, A. 1980. Specific contribution of porcine pancreatic proteinases to *in vitro* digestion of animal and plant-proteins. *Nutrition Reports International,* 22: 771-779.

Gibson, S., McFarlan, C., Hay, S. and MacFarlane, G. 1989. Significance of microflora in proteolysis in the colon. *Applied and Environmental Microbiology,* 55: 679-683.

Gray, V., Kelly, G., Xia, M., Butler, C., Thomas, S. and Mayock, S. 2009. The science of USP 1 and 2 dissolution: present challenges and future relevance. *Pharmaceutical Research,* 26: 1289-1302.

Gregory, P., Mcfadyen, M. and Rayner, D. 2007. Pattern of gastric emptying in the pig: relation to feeding. *British Journal of Nutrition,* 64: 45-58.

Greiner, R. and Konietzny, U. 2006. Phytase for food application. *Food Technology and Biotechnology,* 44: 125-140.

Hamilton, D. and Roe, W. 1977. Electrolyte levels and net fluid and electrolyte movements in the gastrointestinal tract of weanling swine. *Canadian Journal of Complimentary Medicine,* 41: 241-250.

Hedemann, M.S. and Jensen, B. . 2004. Variations in enzyme activity in stomach and pancreatic tissue and digesta in piglets around weaning. *Archives of Animal Nutrition,* 58: 47-59.

Inborr, J., Puhakka, J., Bakker, J. and Meulen, J. 1999. B-glucanase and xylanase activities in stomach and ileum of growing pigs fed wheat bran based diets with and without enzyme treatment. *Archives of Animal Nutrition,* 52: 263-274.

Iraki, L., Bogdan, A., Hakkou, F., Amrani, N., Abkari, A. and Touitou, Y. 1997. Ramadan diet restrictions modify the circadian time structure in humans. A study on plasma gastrin, insulin, glucose, and calcium and on gastric pH. *The Journal of Clinical Endocrinology and Metabolism,* 82: 1261-1273.

Johansen, H.N., Knudsen, K.E.B., Sandström, B. and Skjøth, F. 1996. Effects of varying content of soluble dietary fibre from wheat flour and oat milling fractions on gastric emptying in pigs. *British Journal of Nutrition,* 75: 339-351.

Johnston, N., Dettmar, P., Bishwokarma, B., Lively, M.O. and Koufman, J. 2007. Activity/stability of human pepsin: implications for reflux attributed laryngeal disease. *Laryngoscope,* 117: 1036-1039.

Jongbloed, A., Mroz, Z. and Kemme, P. 1992. The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. *Journal of Animal Science,* 70: 1159-1168.

Karamanolis, G., Theofanidou, I., Yiasemidou, M., Giannoulis, E., Triantafyllou, K. and Ladas, S.D. 2008. A glass of water immediately increases gastric pH in healthy subjects. *Digestive Diseases and Sciences,* 53: 3128-3132.

Keil, B. 1992. *Specificity of Proteolysis*, 1st ed. Springer-Verlag, New York, LLC.

Kemme, P., Schlemmer, U., Mroz, Z. and Jongbloed, A. 2006. Monitoring the stepwise phytate degradation in the upper gastrointestinal tract of pigs. *Journal of the Science of Food and Agriculture,* 86: 612-622.

Van Leeuwen, P. and Jansman, A.J.M. 2007. Effects of dietary water holding capacity and level of fermentable organic matter on digesta passage in various parts of the digestive tract in growing pigs. *Livestock Science,* 109: 77-80.

Létourneau-Montminy, M., Jondreville, C., Sauvant, D. and Narcy, A. 2012. Meta-analysis of phosphorus utilization by growing pigs: effect of dietary phosphorus, calcium and exogenous phytase. *Animal,* 6: 1590-1600.

Low, A. 1982. The activity of pepsin, chymotrypsin and trypsin during 24 h periods in the small intestine of growing pigs. *British Journal of Nutrition,* 48: 147-159.

Luo, H., Wang, Y., Li, J., Wang, H., Yang, J., Yang, Y., Huang, H., Fan, Y. and Yao, B. 2009. Cloning, expression and characterization of a novel acidic xylanase, XYL11B, from the acidophilic fungus Bispora Sp. MEY-1. *Enzyme and Microbial Technology,* 45: 126-133.

Luo, H., Yang, J., Yang, P., Li, J., Huang, H., Shi, P., Bai, Y., Wang, Y., Fan, Y. and Yao, B. 2010. Gene cloning and expression of a new acidic family 7 endo-beta-1,3-1,4-glucanase from the acidophilic fungus Bispora Sp. MEY-1. *Applied Microbiology and Biotechnolgy,* 85: 1015-1023.

Mathew, A., Franklin, M., Upchurch, W. and Chattin, S. 1996. Effect of weaning on ileal short-chain fatty acid concentrations in pigs. *Nutrition Research,* 16: 1689-1698.

Mavromichalis, I., Hancock, J.D., Senne, B.W., Gugle, T.L., Kennedy, G.A., Hines, R.H. and Wyatt, C.L. 2000. Enzyme supplementation and particle size of wheat in diets for nursery and fnishing pigs. *Journal of Animal Science,* 78: 3086-3095.

Molly, K., Woestyne, M., De Smet, I. and Verstraete, W. 1994. Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. *Microbial Ecology in Health and Disease,* 7: 191-200.

Morales, A., García, H., Sánchez, J., Araiza, B., Zijlstra, R. and Cervantes, M. 2012. Apparent ileal amino acid digestibility and activities of trypsin and chymotrypsin in pigs fed sorghum-soybean meal diets supplemented with a microbial phytase. *Animal Feed Science and Technology,* 172: 247-251.

Morgavi, D. and Beauchemin, K. 2001. Resistance of feed enzymes to proteolytic inactivation by rumen microorganisms and gastrointestinal proteases. *Journal of Animal Science,* 79: 1621-1630.

Nyachoti, C. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. *Journal of Animal Science,* 84: 125-134.

Olukosi, O., Sands, J. and Adeola, O. 2007. Supplementation of carbohydrases or phytase individually or in combination to diets for weanling and growing-finishing pigs. *Journal of Animal Science,* 85: 1702-1711.

Omogbenigun, F., Nyachoti, C. and Slominski, B. 2004. Dietary Supplementation with Multienzyme Preparations Improves Nutrient Utilization and Growth Performance in Weaned Pigs. *Journal of Animal Science,* 82: 1053-1061.

Oryschak, M., Simmins, P. and Zijlstra, R. 2002. Effect of dietary particle size and carbohydrase and/or phytase supplementation on nitrogen and phosphorus excretion of grower pigs. *Canadian Journal of Animal Science,* 82: 533-540.

Pandee, P., Summpunn, P., Wiyakrutta, S., Isarangkul, D. and Meevootisom, V. 2011. A thermostable phytase from *Neosartorya spinosa* BCC 41923 and its expression in *Pichia pastoris*. *Journal of Microbiology,* 49: 257-264.

Partanen, K., Jalava, T. and Valaja, J. 2007. Effects of a dietary organic acid mixture and of dietary fibre levels on ileal and faecal nutrient apparent digestibility, bacterial nitrogen flow, microbial metabolite concentrations and rate of passage in the digestive tract of pigs. *Animal,* 1: 389-401.

Peilong, Y., Shi, P., Wang, Y., Bai, Y., Meng, K., Luo, H., Yuan, T. and Yao, B. 2007. Cloning and overexpression of Paenibacillus b-glucanase in *Pichia pastoris*: purification and characterization of the recombinant enzyme. *Journal of Microbiology and Biotechnology,* 17: 58-66.

Potkins, Z., Lawrence, T. and Thomlinson, J. 2007. Effects of structural and non-structural polysaccharides in the diet of the growing pig on gastric emptying rate and rate of passage of digesta to the terminal ileum and through the total gastrointestinal tract. *British Journal of Nutrition,* 65: 391-413.

Rainbird, A.L., Low, A.G. and Zebrowska, T. 1984. Effect of guar gum on glucose and water absorption from isolated loops of jejunum in conscious growing pigs. *British Journal of Nutrition,* 52: 489-498.

Rapp, C., Lantzsch, H. and Drochner, W. 2001. Hydrolysis of phytic acid by intrinsic plant and supplemented microbial phytase (*Aspergillus niger*) in the stomach and small intestine of minipigs fitted with re-entrant cannulas. 1. Passage of dry matter and total phosphorus. *Journal of Animal Physiology and Animal Nutrition,* 85: 406-413.

Rasmussen, L.E. and Meyer, A. S. 2010. Size exclusion chromatography for the quantitative profiling of the enzyme-catalyzed hydrolysis of xylo-oligosaccharides. *Journal of Agricultural and Food Chemistry,* 58: 762-769.

Rasmussen, L.E., Xu, C., Sørensen, J.F., Nielsen, M.K. and Meyer, A.S. 2012. Enzyme kinetics and identification of the rate-limiting step of enzymatic arabinoxylan degradation. *Biochemical Engineering Journal,* 69: 8-16.

Richter, C., Tanaka, T. and Yada, R.Y. 1998. Mechanism of activation of the gastric aspartic proteinases: pepsinogen, progastricsin and prochymosin. *Biochemical Journal,* 335: 481-490.

Ritter, P., Kohler, C. and Von Ah, U. 2009. Evaluation of the passage of *Lactobacillus gasseri* K7 and Bifidobacteria from the stomach to intestines using a single reactor model. *BMC Microbiology,* 9: 87.

Robic, S., Linscott, K.B., Aseem, M., Humphreys, E.A and McCartha, S.R. 2011. Bile acids as modulators of enzyme activity and stability. *Protein Journal,* 30: 539-45.

Rodriguez, E., Mullaney, E. and Lei, X. 2000. Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. *Biochemical and Biophysical Research Communications,* 268: 373-378.

Rodriguez, E., Porres, J., Han, Y. and Lei, X. 1999. Different sensitivity of recombinant *Aspergillus niger* phytase (r-PhyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-AppA) to trypsin and pepsin *in vitro*. *Biochemical and Biophysical Research Communications,* 365: 262-267.

Roy, M.P., Poddar, M., Singh, K.K. and Ghosh, S. 2012. Purification, characterization and properties of phytase from Shigella Sp. CD2. *Indian Journal of Biochemistry and Biophysics,* 49: 266-271.

Scharschmidt, B. F., Keeffe, E.B., Vessey, D.a, Blankenship, N.M. and Ockner, R.K. 1981. *In vitro* effect of bile salts on rat liver plasma membrane, lipid fluidity, and ATPase activity. *Hepatology,* 1: 137-145.

Schecter, I. and Berger, A. 1967. On the size of the active site in proteases. I. Papain. *Biochemical and Biophysical Research Communications,* 27: 157-162.

Schulze, F. and Jacob, H. 1981. Redox potential in gastrointestinal-tract of swine, different age-groups. *Archiv für experimentelle Veterinärmedizin,* 35: 349-357.

Selle, P.H., Ravindran, V., Caldwell, A. and Bryden, W.L. 2000. Phytate and phytase: consequences for protein utilisation. *Nutrition Research Reviews,* 13: 255-278.

Sena, A.R., Júnior, G.L.V, Neto, A.G. and Taranto, A.G. 2011. Production, purification and characterization of a thermostable B-1,3-glucanase (laminarinase) produced by *Moniliophthora perniciosa*. *Annals of the Brazilian Academy of Sciences,* 83: 599-609.

Siew, L.F., Basit, A.W. and Newton, J.M. 2000. The potential of organic-based amylose-ethylcellulose film coatings as oral colon-specific drug delivery systems. *AAPS PharmSciTech,* 1: E22.

Sipos, T. and Merkel, J. R. 1970. An effect of calcium ions on the activity, heat stability, and structure of trypsin. *Biochemistry,* 9: 2766-2775.

Snoeck, V., Huyghebaert, N., Cox, E., Vermeire, A., Saunders, J., Remon, J., Verschooten, F. and Goddeeris, B. 2004. Gastrointestinal transit time of nondisintegrating radio-opaque pellets in suckling and recently weaned piglets. *Journal of Controlled Release,* 94: 143-153.

Solà-Oriol, D., Torrallardona, D. and Gasa, J. 2010. Role of dietary fibre source and meal size on the ileal transit of digesta in growing pigs. *Livestock Science* 133: 67-69.

Sumeri, I., Arike, L., Adamberg, K. and Paalme, T. 2008. Single bioreactor gastrointestinal tract simulator for study of survival of probiotic bacteria. *Applied Microbiology and Biotechnolgy,* 80: 317-324.

Thacker, P.A. and Baas, T.C. 1996. Effects of gastric pH on the activity of exogenous pentosanase and the effect of pentosanase supplementation of the diet on the performance of growing-finishing pigs. *Animal Feed Science and Technology,* 63: 187-200.

The United States Pharmacopeial Convention. 2007. *United States Pharmacopeia and National Formulary USP 29- NF 24. Inc.* Rockville, MD.

Uribe, S. and Sampedro, J.G. 2003. Measuring solution viscosity and its effect on enzyme activity. *Biological Procedures Online,* 5: 108-115.

Wainø, M. and Ingvorsen, K. 2003. Production of beta-xylanase and beta-xylosidase by the extremely halophilic archaeon halorhabdus utahensis. *Extremophiles,* 7: 87-93.

Wang, M.Q., Xu, Z.R., Sun, J.Y. and Kim, B.G. 2008. Effects of enzyme supplementation on growth, intestinal content viscosity, and digestive enzyme activities in growing pigs fed rough rice-based diet. *Asian-Australian Journal of Animal Science,* 21: 270-276.

Wilfart, A., Montagne, L., Simmins, H., Noblet, J. and Van Milgen, J. 2007. Effect of fibre content in the diet on the mean retention time in different segments of the digestive tract in growing pigs. *Livestock Science,* 109: 27-29.

Wilson, R.H. and Leibholz, J. 1981. Digestion in the pig between 7 and 35 d of age. 2. The digestion of dry matter and the pH of digesta in pigs given milk and soya-bean proteins. *British Journal of Nutrition,* 45: 321-336.

Wong D., Larrabee S., Clifford K., Tremblay J. and Friend D.R. 1997. USP dissolution apparatus III (reciprocating cylinder) for screening of guar-based colonic delivery formulations. *Journal of Controlled Release*. 47: 173-179.

Woyengo, T.A., Adeola, O., Udenigwe, C.C. and Nyachoti, C.M. 2010. Gastro-intestinal digesta pH, pepsin activity and soluble mineral concentration responses to supplemental phytic acid and phytase in piglets. *Livestock Science,* 134: 91-93.

Wyss, M., Pasamontes, L., Friedlein, A., Tessier, M., Kronenberger, A., Lehmann, M., Schnoebelen, L. *et al.,* 1999. Biophysical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. *Applied Environmental Microbiology,* 65: 359-366.

Yáñez, J. L., Beltranena, E., Cervantes, M. and Zijlstra, R. T. 2011. Effect of phytase and xylanase supplementation or particle size on nutrient digestibility of diets containing distillers dried grains with solubles cofermented from wheat and corn in ileal-cannulated grower pigs. *Journal of Animal Science,* 89: 113-123.

Yi, Z. and Kornegay, E. T. 1996. Sites of phytase activity in the gastrointestinal tract of young pigs. *Animal Feed Science and Technology,* 61: 361-368.

Yin, Y.L., Baidoo, S., Jin, L., Liu, Y., Schulze, H. and Simmins, P. 2001. The effect of different carbohydrase and protease supplementation on apparent (ileal and overall) digestibility of nutrients of five hulless barley varieties in young pigs. *Livestock Production Science,* 71: 109-120.

Zhang, G., Dong, X., Wang, Z., Zhang, Q., Wang, H. and Tong, J. 2010. Purification, characterization, and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from *Aspergillus ficuum* NTG-23. *Bioresource Technology,* 101: 4125-4131.

Zhao, J., Shi, P., Yuan, T., Huang, H., Li, Z., Meng, K., Yang, P. and Yao, B. 2012. Purification, gene cloning and characterization of an acidic B-1,4-glucanase from *Phialophora* Sp. G5 with potential applications in the brewing and feed industries. *Journal of Bioscience and Bioengineering,* 114: 379-84.

Zhu, W., Qiao, D., Huang, M., Yang, G., Xu, H. and Cao, Y. 2010. Modifying thermostability of appA from *Escherichia coli*. *Current Microbiology,* 61: 267-273.

Animal Nutrition and Feed Technology (2013) 13: 461-476

In Vitro Evaluation of Commercial Fibrolytic Enzymes for Improving the Nutritive Value of Low-Quality Forages[#]

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ABSTRACT

Díaz, A., Carro, M.D., Saro, C., Mateos, I., Odongo, E. and Ranilla, M.J. 2013. In vitro evaluation of commercial fibrolytic enzymes for improving the nutritive value of low-quality forages. Animal Nutrition and Feed Technology, 13: 461-476.

The aim of this work was to assess the effects of four doses of three commercial fibrolytic enzymes on ruminal fermentation of rice straw, maize stover and *Pennisetum purpureum* clon Cuba CT-115 hay in batch cultures of ruminal micro-organisms from sheep. One enzyme was produced by *Penicillium funiculosum* (PEN) and two were from *Trichoderma longibrachiatum* (TL1 and TL2). Each liquid enzyme was diluted 200 (D1), 100 (D2), 50 (D3) and 10 (D4) - fold and applied to each substrate in quadruplicate over time and incubated for 120 h in rumen fluid. The D4 dose of each enzyme increased ($P<0.05$) the fractional rate of gas production and organic matter effective degradability for all substrates, and TL2 had similar effects when applied at D3. In 9 h incubations, PEN at D4, TL1 at all tested doses, and TL2 at D2, D3 and D4 increased ($P<0.05$) volatile fatty acid production and dry matter degradability for all substrates. The commercial enzymes tested were effective at increasing *in vitro* ruminal fermentation of low-quality forages, although effective doses varied with the enzyme.

Key words: Fibrolytic enzymes, Low-quality forages, Rumen fermentation, Batch cultures.

INTRODUCTION

One of the main constraints for increasing ruminant production in developing countries is inadequate nutrition. Feed resources in such countries are characterized by scarcity during the winter or dry season and fluctuating quality of forage, resulting in nutrient imbalance when ruminants are grazed or fed the available pastures and crop residues. The problem is worsened by the limited usage of commercial concentrate feeds due to high costs. The situation is particularly acute in the dry season when high-quality forages are in short supply and nutrient requirements of ruminants cannot be met

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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(Leng, 1990). Farmers usually try to counter this shortfall by conserving forages and crop residues, such as wheat and rice straw or maize stover, but most of these forages are high in fiber and low in protein, which limits animal performance. Therefore, any improvements made in their nutrient utilization will be expected to increase ruminant productivity and reduce manure output.

Among the techniques available to improve the nutritive value of low-quality forages, treatment with fibrolytic enzymes (ENZ) is one of the less explored, because most studies on fibrolytic ENZ have been conducted with medium to good-quality forages. In the last decades the cost of production of exogenous ENZ has considerably decreased (McAllister *et al.*, 2000), and currently there are different commercial products whose use at the concentrations necessary to produce a positive animal response can be economically feasible. However, the effectiveness of fibrolytic ENZ is influenced by many factors such as type and dose of ENZ and type of diet fed to animals. Therefore ENZ should be tested on different forages at different doses to identify the optimum ENZ dose for a particular forage. The aim of this study was to assess the effects of different doses of three commercial ENZ preparations on the *in vitro* ruminal fermentation of three low-quality forages. Our hypothesis was that each enzyme would increase the fermentation of the forages but effects of each commercial product would depend on the type of forage tested.

MATERIALS AND METHODS

Substrates and enzymes

Samples of rice straw (*Oriza sativa L.*), maize stover (*Zea mays*) and a tropical grass hay (*Pennisetum purpureum* clon Cuba CT-115) were selected as substrates to be tested, as they represent the main forage source used in ruminant production systems in many tropical countries. The chemical composition of the forages is given in Table 1.

Table 1. Chemical composition (g/kg dry matter) of forages used in the study

	Organic matter	*Crude protein*	*Neutral detergent fibre*	*Acid detergent fibre*	*Acid detergent lignin*
Rice straw	856	52.4	729	465	58.0
Maize stover	955	30.8	856	456	43.7
Pennisetum purpureum clon Cuba CT-115 hay	885	62.6	699	381	62.9

Three commercial ENZ preparations were selected: Rovabio Excel® (PEN; produced by *Penicillium funiculosum;* Adisseo, Antony, France) and Xylanase Plus (TL1) and Cellulase Plus (TL2) produced by *T. longibrachiatum* (Dyadic®, Jupiter, FL, USA). Enzyme preparations were assayed for endoglucanase, exoglucanase, xylanase and amylase activities using carboxymethylcellulose, Avicel PH-101, oat spelt xylan and soluble starch as substrates, respectively. In order to simulate ruminal conditions in

forage-fed ruminants, all ENZ activities were measured at pH 6.5 and 39°C following the procedures described by Giraldo *et al.* (2008a). Determinations were done in triplicate, and tubes containing only buffer, buffer plus substrate, and buffer plus ENZ were also incubated to correct for substrate autolysis and sugars present in the ENZ preparations. Exactly, 1 mL of PEN liberated 7.5 μmol of glucose from carboxymethyl-cellulose and 613 μmol of xylose from oat spelt xylan per min, and TL1 and TL2 liberated 19.5 μmol of glucose from carboxymethylcellulose and 661 μmol of xylose from oat spelt xylan per min, and 16.6 μmol of glucose from carboxymethyl-cellulose and 690 μmol of xylose from oat spelt xylan per min. No exoglucanase or amylase activities were detected for any ENZ.

In vitro gas production kinetics and in vitro ruminal fermentation

Samples of forages were ground through a 1-mm screen before *in vitro* incubations. Four hundred mg of each forage were accurately weighed into 120-mL serum bottles. Enzyme preparations were supplied in liquid form and they were diluted 1/200 (D1), 1/100 (D2), 1/50 (D3) and 1/10 (D4) in 0.1 M sodium phosphate buffer (pH= 6.5). One mL of the corresponding solution was carefully applied directly onto the substrate inside the bottles 24 h before starting the incubation, and bottles were kept at 21-23°C until incubation. Enzyme activities added to forages varied among the commercial products (Table 2), but were comparable to those used in previous studies (Eun and Beauchemin, 2007; Giraldo *et al.*, 2008ab; Ranilla *et al.*, 2008).

Table 2. Endoglucanase and xylanase activity added to forage samples (400 mg of dry matter) in each experimental treatment†

Enzyme	Endoglucanase				Xylanase			
	D1	D2	D3	D4	D1	D2	D3	D4
PEN	0.04	0.08	0.15	0.75	3.07	6.13	12.3	61.3
TL1	0.10	0.20	0.39	1.95	3.31	6.61	13.2	66.1
TL2	0.08	0.17	0.33	1.66	3.45	6.90	13.8	69.0

†Endoglucanase and xylanase activities are expressed as μmol of glucose and xylose, respectively, released by 1 mL of enzyme per minute at 39°C and pH 6.5

Ruminal fluid was obtained from four rumen-cannulated Merino sheep fed medium quality grass hay at energy maintenance level (NRC, 2007). Sheep were managed according to the protocols approved by the León University Institutional Animal Care and Use Committee, and had free access to water and mineral/vitamin blocks throughout the trial. Ruminal contents of each sheep were obtained immediately before the morning feeding, mixed and strained through four layers of cheese cloth into an Erlenmeyer flask with an O_2-free headspace. The fluid was mixed with the buffer solution of Goering and Van Soest (1970; no trypticase added) in a 1:4 (v/v) proportion at 39°C under continuous flushing with CO_2. Bottles were prewarmed (39°C) prior to the addition of 40 ml of buffered rumen contents under CO_2 flushing. Then, bottles were sealed with rubber stoppers & aluminium caps & incubated at 39°C.

Bottles used to analyse ENZ effects on gas production kinetics were incubated for 120 h. Gas production was measured at 3, 6, 9, 12, 18, 24, 36, 48, 72, 96 and 120 h using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a calibrated syringe and the gas was released after each measurement. After 120 h of incubation, the fermentation was stopped by swirling the bottles in iced water, the bottles were opened and their contents were transferred to previously weighed filter crucibles (pore size 100-160 μm) and filtered under vacuum. The incubation residues were washed with 50 ml of hot distilled water, dried at 50°C for 48 h, and analysed for ash to calculate apparent organic matter (OM) disappearance after 120 h of incubation (OMD_{120}). The incubations were repeated 3 times, so that each treatment was evaluated in quadruplicate. In each incubation, additional bottles without substrate (blanks) but with the corresponding ENZ treatment were included to correct the gas production values for gas release from endogenous substrates and ENZ treatment (Carro *et al.*, 2005).

Bottles used to analyse ENZ effects on ruminal fermentation were incubated for 9 h. Gas production was measured as before described at 3, 6 and 9 h. Bottles were uncapped, the pH immediately measured with a pH-meter (Crison pH Meter Basic 20, Barcelona, Spain) and the fermentation was stopped by swirling the bottles in iced water. Samples were taken for volatile fatty acid (VFA) and ammonia-N analysis as described by Giraldo *et al.* (2008a), and the contents of the bottles were transferred to previously weighed filter crucibles to calculate DM degradability (DMD). Each incubation was repeated 3 times to give 4 replicates per treatment, and blanks were included in each incubation series.

The effects of ENZ treatments on substrate fibre composition were analysed by weighing 400 mg of forage samples into artificial fibre bags (#F57 bags; 50 x 40 mm; 25±10 μm pore size; ANKOM Technology Corporation, Fairport, USA) and adding 1 mL of the different ENZ dilutions tested into each bag. Bags were heat sealed and kept at room temperature (21-23°C) for 24 h before sequential neutral detergent fibre (NDF) and acid detergent fibre (ADF) analyses were conducted. For replication, the complete procedure was repeated 3 times.

Analytical procedures

Dry matter, ash and N were determined according to methods of the Association of Official Analytical Chemists (1999), NDF and ADF concentrations were measured as described by Giraldo *et al.* (2008a), and VFA and NH_3-N were analysed as described by Carro and Miller (1999).

Calculations and statistical analyses

Gas production data were fitted using the exponential model: $gas = A\ [1 - e\ \{-c\ (t - lag)\}]$, where A is the asymptotic gas production, c is the fractional rate of gas production, lag is the initial delay in the onset of gas production and t is the gas

measurement time. The parameters *A*, *c* and *lag* were estimated by an iterative least squares procedure using the NLIN procedure of SAS (2012). Halftime of gas production ($t_{1/2}$; time when half of the asymptotic gas volume is produced) was calculated as $t_{1/2}=[(\ln 2/c) + lag]$. The average fermentation rate (AFR; average gas production rate between the start of the incubation and $t_{1/2}$) was calculated as $AFR=A\ c/[2 (\ln 2 + c\ lag)]$ and OM effective degradability (OMED) was estimated assuming a rumen particulate outflow rate (*Kp*) of 0.035 per h, which is characteristic of sheep fed forages at the maintenance level (Ranilla *et al.*, 1998), according to the following equation: $OMED=[(OMD_{120}\ c)/ (c + Kp)]\ e\ (^{-\ c\ lag})$. In the *in vitro* fermentation trial, the amounts of VFA produced were obtained by subtracting the amount present initially in the incubation medium from that determined after 9 h of incubation.

Data were analysed separately for each ENZ treatment. Five ENZ doses, three forages, and the interaction of ENZ dose×forage were included in the model as fixed effects, whereas incubation day was considered as a random effect. Each ENZ dose l x forage interaction mean had four replicates. Non-orthogonal polynomial contrasts were used to test for linear, quadratic, and cubic effects of ENZ. The MIXED procedure of SAS (2012) was used for all statistical analyses. Significance was declared at $P<0.05$, whereas $P>0.5<0.10$ values were considered to be a trend. When a significant effect of ENZ or an ENZ×forage interaction was detected, each ENZ treatment mean was compared with the corresponding control using Dennett's test.

RESULTS

The effects of different doses of PEN, TL1 and TL2 on *in vitro* gas production kinetics of forages are shown in Tables 3, 4 and 5, respectively. A cubic effect ($P<0.001$) of ENZ levels was observed for most of determined gas production parameters, with the exception of *A* parameter for rice straw and maize stover and OMD_{120} for all forages, which were not affected ($P>0.05$) by any ENZ treatment. Relative to the control, all PEN doses increased ($P<0.05$) parameter *c* and OMED and decreased ($P>0.05$) $t_{1/2}$ for grass hay, but these effects were only observed with D4 for maize stover and rice straw. Applied at D4, TL1 increased ($P<0.05$) parameter *c* and OMED and decreased ($P>0.05$) $T_{1/2}$ for all forages, but no effects ($P>0.05$) of D1, D2 and D3 were observed with any forage (Table 4). With grass hay, TL2 at D2, D3 and D4 increased ($P<0.05$) parameter *c* and decreased ($P>0.05$) $t_{1/2}$, whereas only D3 and D4 doses were effective ($P<0.05$) with maize stover and D4 with rice straw.

Treatment of forages with all ENZ had a cubic effect ($P<0.001$) on NDF and ADF content (Table 6). When the effects of each ENZ were analysed individually, there were no ENZxforage interactions ($P=0.147$ to 0.730). However, there were differences between ENZ when they were applied to the same forage. For grass hay, TL1 was the most effective, as all tested doses decreased ($P<0.05$) NDF and ADF content, and only D4 dose of PEN and TL2 reduced ($P<0.05$) ADF content. For rice straw, all doses of TL2 decreased ($P<0.05$) NDF and ADF content, but only D3 and D4 doses of PEN

and TL1 had similar effects. All doses of TL1 decreased ($P<0.05$) NDF content of maize stover, but no effects ($P=0.05$) of D1 were observed for PEN and TL2.

Table 3. Effect of 24-h pre-treatment with increasing doses (D1, D2, D3 and D4) of PEN enzyme (Rovabio Excel®, Adisseo) on *in vitro* gas production kinetics of tropical grass (*Pennisetum purpureum* clon Cuba CT-115) hay, maize stover and rice straw in batch cultures of ruminal micro-organisms (n=4)

Substrate and PEN Dose[2]	*In vitro* gas production kinetics[1]					
	A	c	AFR	$t_{1/2}$	OMD_{120}	OMED
Tropical grass hay						
CON	158	0.019	2.19	36.4	57.8	22.4
D1	161	0.023*	2.67*	30.6*	57.2	24.6*
D2	161	0.023*	2.63*	30.8*	56.5	24.3*
D3	161	0.022*	2.57†	31.5*	56.0	24.1*
D4	172†	0.025*	3.10*	28.0*	56.7	25.7*
Maize stover						
CON	208	0.022	3.21	32.9	70.2	28.8
D1	207	0.023	3.47	30.8	71.7	30.0
D2	213	0.023	3.41	31.5	70.4	29.7
D3	211	0.023	3.39	31.4	68.2	28.8
D4	221	0.025*	3.91*	28.4*	68.3	30.7*
Rice straw						
CON	166	0.020	2.44	35.1	52.1	20.7
D1	157	0.021	2.33	33.6	53.7	21.9
D2	156	0.021	2.33	33.6	53.3	21.7
D3	157	0.021	2.43	32.4†	52.6	21.9
D4	167	0.026*	3.13*	26.8*	52.8	24.5*
SEM[3]	5.2	0.0008	0.153	0.99	0.93	0.52
P values						
Substrate	<0.001	0.008	<0.001	0.081	<0.001	<0.001
Substrate x enzyme	0.894	0.071	0.709	0.096	0.619	0.271
Enzyme:						
Linear effect	0.141	0.550	0.505	0.211	0.722	0.572
Quadratic effect	0.484	<0.001	0.005	<0.001	0.254	<0.001
Cubic effect	0.019	<0.001	<0.001	<0.001	0.188	0.003

*For each substrate and variable, means differ from control ($P<0.05$). †For each substrate and variable, means differ from control ($P<0.10$)

[1]*A*: asymptotic gas production (mL/0.4 g); *c*: fractional rate of fermentation (h^{-1}); AFR: average fermentation rate (mL/h); $T_{1/2}$: half time of gas production (h); OMED: organic matter effective degradability (%) for a fractional passage rate of 0.035 h^{-1}; $OMED_{120}$: organic matter degradability (%) after 120 h of incubation

[2]CON: control; D1, D2, D3 and D4 correspond to 1/200, 1/100, 1/50 and 1/10 dilutions of PEN.

[3]Standard error of the mean

Table 4. Effect of 24-h pre-treatment with increasing doses (D1, D2, D3 and D4) of TL1 enzyme (Xylanase Plus Dyadic®) on *in vitro* gas production kinetics of tropical grass (*Pennisetum purpureum* clon Cuba CT-115) hay, maize stover and rice straw in batch cultures of ruminal micro-organisms (n=4)

Substrate and TL1 Dose[2]	In vitro gas production kinetics[1]					
	A	c	AFR	$t_{1/2}$	OMD_{120}	OMED
Tropical grass hay						
CON	158	0.019	2.19	36.4	57.8	22.4
D1	159	0.019	2.20	36.2	58.1	22.6
D2	161	0.019	2.27	35.9	57.2	22.5
D3	160	0.020	2.36	34.0	57.5	23.3
D4	164	0.024*	2.82*	29.4*	57.8	25.5*
Maize stover						
CON	208	0.022	3.21	32.9	70.2	28.8
D1	210	0.022	3.24	32.8	70.5	29.1
D2	210	0.022	3.25	32.6	70.7	29.2
D3	213	0.022	3.26	33.1	70.0	28.7
D4	219	0.025*	3.81*	29.1*	70.3	31.2*
Rice Straw						
CON	166	0.020	2.44	35.1	52.1	20.7
D1	167	0.020	2.44	34.2	52.4	21.2
D2	163	0.020	2.39	34.2	51.9	20.9
D3	167	0.020	2.44	34.2	52.0	21.0
D4	173	0.023*	2.81†	30.9*	52.5	22.5*
SEM[3]	5.5	0.0008	0.150	1.05	1.06	0.63
P values						
Substrate	<0.001	<0.001	<0.001	0.005	<0.001	<0.001
Substrate x enzyme	0.999	0.665	0.994	0.674	0.999	0.927
Enzyme:						
Linear effect	0.812	0.234	0.187	0.183	0.897	0.202
Quadratic effect	0.649	0.086	0.169	0.057	0.772	0.075
Cubic effect	0.107	<0.001	<0.001	<0.001	0.811	<0.001

*For each substrate and variable, means differ from control ($P<0.05$). †For each substrate and variable, means differ from control ($P<0.10$)

[1]*A*: asymptotic gas production (mL/0.4 g); *c*: fractional rate of fermentation (h^{-1}); AFR: average fermentation rate (mL/h); $T_{1/2}$: half time of gas production (h); OMED: organic matter effective degradability (%) for a fractional passage rate of 0.035 h^{-1}; $OMED_{120}$: organic matter degradability (%) after 120 h of incubation

[2] CON: control; D1, D2, D3 and D4 correspond to 1/200, 1/100, 1/50 and 1/10 dilutions of **TL1**.

[3]Standard error of the mean

Table 5. Effect of 24-h pre-treatment with increasing doses (D1, D2, D3 and D4) of TL2 enzyme (Xylanase Plus Dyadic®) on *in vitro* gas production kinetics of tropical grass (*Pennisetum purpureum* clon Cuba CT-115) hay, maize stover and rice straw in batch cultures of ruminal micro-organisms (n=4)

Substrate and TL2 Dose[2]	In vitro gas production kinetics[1]					
	A	c	AFR	$t_{1/2}$	OMD_{120}	OMED
Tropical grass hay						
CON	158	0.019	2.19	36.4	57.8	22.4
D1	158	0.021†	2.38	33.5*	57.6	23.5
D2	161	0.021*	2.47	33.0*	57.1	23.6
D3	152	0.022*	2.46	31.0*	55.9†	23.9†
D4	160	0.028*	3.23*	24.7*	57.1	27.6*
Maize stover						
CON	208	0.022	3.21	32.9	70.2	28.8
D1	209	0.022	3.26	32.4	70.5	29.3
D2	207	0.022	3.26	32.0	69.1	29.0
D3	205	0.024†	3.50	29.7*	70.1	30.7*
D4	211	0.027*	4.10*	26.1*	69.5	32.7*
Rice Straw						
CON	166	0.020	2.44	35.1	52.1	20.7
D1	163	0.020	2.35	34.9	53.9†	21.5
D2	156	0.022†	2.44	32.1*	54.7*	22.9*
D3	160	0.023*	2.62	30.4*	52.9	22.8*
D4	168	0.028*	3.43*	24.6*	55.3*	26.8*
SEM[3]	5.1	0.007	0.124	0.88	0.71	0.56
P values						
Substrate	<0.001	0.029	<0.001	0.125	<0.001	<0.001
Substrate x enzyme	0.964	0.075	0.918	0.201	0.094	0.403
Enzyme:						
Linear effect	0.167	0.313	0.065	0.854	0.601	0.532
Quadratic effect	0.594	0.001	0.007	<0.001	0.099	<0.001
Cubic effect	0.903	<0.001	<0.001	<0.001	0.747	<0.001

*For each substrate and variable, means differ from control ($P<0.05$); †For each substrate and variable, means differ from control ($P<0.10$)

[1]*A*: asymptotic gas production (mL/0.4 g); *c*: fractional rate of fermentation (h^{-1}); AFR: average fermentation rate (mL/h); $T_{1/2}$: half time of gas production (h); OMED: organic matter effective degradability (%) for a fractional passage rate of 0.035 h^{-1}; $OMED_{120}$: organic matter degradability (%) after 120 h of incubation

[2]CON: control; D1, D2, D3 and D4 correspond to 1/200, 1/100, 1/50 and 1/10 dilutions of **TL2**.

[3]Standard error of the mean

Tables 7, 8 and 9 show the effects of PEN, TL1 and TL2 on *in vitro* fermentation parameters of forages, respectively. There were no effects of ENZ ($P>0.05$) on final pH and the results are not shown. For all tested ENZ, a cubic effect ($P<0.001$) of ENZ dose was observed for gas production, DMD, total VFA production, molar proportions of acetate, propionate and butyrate, and acetate: propionate (Ac: Pr) ratio. Whereas no ENZ x forage interactions ($P>0.05$) for NH_3-N concentrations, DMD and VFA production were detected with PEN and TL2, ENZ x forage interactions were observed

Table 6. Effect of 24-h pre-treatment with increasing doses (D1, D2, D3 and D4) of commercial enzymes (PEN, TL1 and TL2)† on NDF and ADF content (g/kg DM) of tropical grass (*Pennisetum purpureum* clon Cuba CT-115) hay, maize stover and rice straw (n=4)[1]

	Tropical grass hay					Maize Stover					Rice Straw					SEM[3]	P values[2]				
	CON	D1	D2	D3	D4	CON	D1	D2	D3	D4	CON	D1	D2	D3	D4		SUB	SUB x ENZ	L	Q	C
PEN																					
NDF	699	82*	85†	679*	671*	856	850	837*	840*	815*	729	725	723	705*	705*	5.6	<0.001	0.298	0.073	0.034	<0.001
ADF	380	381	378	371†	368*	456	456	453	455	444*	465	460	458	445*	439*	4.5	<0.001	0.546	0.562	0.496	<0.001
TL1																					
NDF	699	675*	667*	674*	635*	856	820*	812*	771*	741*	729	714	707	693*	668*	12.7	<0.001	0.268	0.076	0.011	<0.001
ADF	381	374*	367*	375*	355*	456	447	445	407*	409*	465	464	450	432*	425*	12.6	<0.001	0.730	0.380	0.520	<0.001
TL2																					
NDF	699	677*	677*	672*	662*	856	837	829*	828*	788*	729	698*	700*	680*	651*	7.8	<0.001	0.229	0.029	<0.001	<0.001
ADF	380	370	371	366	359*	456	449	450	450	426*	465	437*	442*	433*	404*	6.1	<0.001	0.147	0.259	0.003	<0.001

*For each substrate and variable, means differ from control (P<0.05). † For each substrate and variable, means differ from control (P<0.10)

[1]CON: control; D1, D2, D3 and D4 correspond to 1/200, 1/100, 1/50 and 1/10 dilutions of commercial enzymes, respectively. See text for description of enzymes.

[2]SUB: substrate; ENZ: enzyme; L: linear effect of ENZ dose; Q: quadratic effect of ENZ dose; C: cubic effects of ENZ dose.

[3]Standard error of the mean

Table 7. Effect of increasing doses (D1, D2, D3 and D4) of PEN enzyme (Rovabio Excel®, Adisseo) on gas production after 3, 6 and 9 h, ammonia-N concentration, dry matter degradability (DMD), total volatile fatty acid (VFA) production, molar proportions of VFA, and acetate/propionate ratio (Ac/Pr) after 9 h *in vitro* fermentation of samples (400 mg DM) of *Pennisetum purpureum* clon Cuba CT-115 hay (tropical grass hay), maize stover and rice straw in batch cultures of ruminal microorganisms (n=4)

Substrate	PEN dose[4]	Gas (mL)			NH_3-N	DMD	VFA	Molar proportion (mol/100 mol)[1]				Ac: Pr
		3 h	6 h	9 h	(mg/L)	(%)	(μmol)	Ac	Pr	But	Others	(mol/mol)
Tropical	C	21.9	31.3	37.6	203	16.2	422	72.8	20.9	4.44	1.80	3.60
grass hay	D1	22.0	31.9	37.4	204	18.4	441	72.0	21.9	4.85	1.27	3.44
	D2	22.0	32.1	38.3	204	18.6	461	70.7	22.9	5.18	1.25	3.28
	D3	21.9	32.8	39.9*	197	20.9*	468	70.1	22.7	5.63	1.56	3.23
	D4	24.3*	36.2*	45.1*	196	23.5*	611*	67.7	24.2†	6.82*	1.32	2.91*
Maize	C	21.6	27.3	30.9	205	6.8	230	76.4	20.0	2.35	1.25	3.93
stover	D1	20.5	26.8	31.3	189	8.8	288†	76.9	17.3	2.73	2.98	4.53
	D2	22.1	28.9†	34.2*	186	7.8	317*	75.6	18.5	3.27	2.57	4.23
	D3	21.4	28.9†	34.3*	178†	9.1	365*	71.9	21.7	4.81*	1.64	3.58
	D4	23.3	32.8*	40.3*	171*	13.0*	437*	60.7*	28.6*	8.93*	1.77	2.28*
Rice straw	C	18.5	25.5	28.2	183	10.4	157	70.2	21.8	2.76	5.27	3.49
	D1	16.6†	22.1*	25.6*	175	11.0	175	70.5	18.8	2.48	8.25	3.97
	D2	16.7†	22.8*	26.5*	178	12.4	177	70.0	20.0	2.87	7.12	3.85
	D3	16.7†	21.5*	25.3*	167	11.9	210	70.9	21.0	4.10	3.98	3.75
	D4	17.8	24.8	30.2	134*	14.9*	327*	69.3	20.9	7.72*	2.09	3.58
SEM[2]		0.65	0.64	0.85	10.2	1.09	23.7	2.36	1.22	0.741	1.447	0.229
P values[3]												
SUB		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.340	0.038	0.015	<0.001	0.006
SUB x ENZ		0.445	<0.001	0.004	0.599	0.803	0.723	0.049	0.003	0.144	0.535	0.003
L		0.004	<0.001	0.002	0.597	0.509	0.985	0.500	0.128	0.407	0.528	0.079
Q		0.710	0.082	0.007	0.360	0.024	0.003	0.340	0.824	0.104	0.368	0.857
C		0.004	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	0.054	<0.001

*For each substrate and variable, means differ from control ($P<0.05$). † For each substrate and variable, means differ from control ($P<0.10$)

[1] Ac: acetate; Pr: propionate; But: butyrate; Others: sum of isobutyrate, isovalerate and valerate

[2] Standard error of the mean

[3] SUB: substrate; ENZ: enzyme; L, Q and C: linear, quadratic and cubic effect of ENZ dose, respectively

[4] CON: control; D1, D2, D3 and D4 correspond to 1/200, 1/100, 1/50 and 1/10 dilutions of commercial enzymes, respectively. See text for description of enzymes.

Table 8. Effect of increasing doses of TL1 enzyme on gas production, NH_3-N, DM degradability (DMD), VFA production after 9 h *in vitro* fermentation of samples of tropical grass hay, maize stover and rice straw in batch cultures of ruminal microorganisms (n=4)

Substrate	TL1 dose[4]	Gas (mL)			NH_3-N	DMD	VFA	Molar Proportion (mol/100 mol)[1]				Ac: Pr
		3 h	6 h	9 h	(mg/L)	(%)	(μmol)	Ac	Pr	But	Others	(mol/mol)
Tropical	C	21.9	31.3	37.6	203	16.2	422	72.8	20.9	4.44	1.80	3.60
grass hay	D1	22.6	33.9	43.3†	190	18.5	526*	72.3	21.8	4.78	1.13	3.44
	D2	24.1	35.8*	42.2*	189	20.2*	536*	71.3	22.6*	5.04	1.07	3.26†
	D3	22.9	36.3*	40.3*	191	22.6*	590*	70.0*	23.7*	5.28†	1.06	3.06*
	D4	24.3*	41.3*	51.4*	206	23.6*	808*	65.8*	25.9*	6.17*	2.16	2.62*
Maize	C	21.6	27.3	30.9	205	6.8	230	76.4	20.0	2.35	1.25	3.93
stover	D1	22.2	30.5*	35.4*	184	13.1*	383*	71.5*	22.0*	4.65*	1.79	3.37*
	D2	24.1*	33.4*	38.9*	180	14.0*	457*	70.5*	22.9*	4.97*	1.67	3.21*
	D3	24.1*	34.5*	40.9*	176	15.3*	518*	69.2*	24.5*	5.43*	0.95	2.93*
	D4	24.3*	40.9*	50.6*	183	20.0*	774*	66.2*	25.9*	6.32*	1.53	2.62*
Rice straw	C	18.5	25.5	28.2	183	10.4	157	70.2	21.8	2.76	5.27	3.49
	D1	18.1	24.5	28.5	175	13.6†	204†	73.3*	17.7*	2.80	6.26	4.35*
	D2	18.6	25.4	29.9	159	17.6*	260*	72.7*	18.8*	4.08*	4.72	4.11*
	D3	18.9	26.6	30.6	157	16.7*	311*	72.8*	19.9*	5.22*	2.09	3.85*
	D4	21.2*	33.6*	41.1*	185	17.6*	603*	67.5*	23.8*	6.21*	2.54	2.95*
SEM[2]		0.84	1.10	1.08	11.1	1.20	16.6	0.90	0.25	0.321	0.934	0.129
P values[3]												
SUB		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.389	<0.001	<0.001	<0.001	<0.001
SUB x ENZ		0.555	0.158	0.035	0.262	0.379	<0.001	<0.001	<0.001	0.001	0.268	<0.001
L		0.909	0.918	0.658	0.202	<0.001	0.110	0.576	0.084	<0.001	0.288	0.373
Q		0.093	<0.001	<0.001	0.304	<0.001	<0.001	0.004	0.111	<0.001	0.368	0.156
C		<0.001	<0.001	<0.001	0.585	<0.001	<0.001	<0.001	<0.001	<0.001	0.055	<0.001

* For each substrate and variable, means differ from control ($P<0.05$). † For each substrate and variable, means differ from control ($P<0.10$)

[1] Ac: acetate; Pr: propionate; But: butyrate; Others: sum of isobutyrate, isovalerate and valerate

[2] Standard error of the mean

[3] SUB: substrate; ENZ: enzyme; L, Q and C: linear, quadratic and cubic effect of ENZ dose, respectively

[4] CON: control; D1, D2, D3 and D4 correspond to 1/200, 1/100, 1/50 and 1/10 dilutions of commercial enzymes, respectively. See text for description of enzymes.

Table 9. Effect of increasing doses (D1, D2, D3 and D4) of TL2 enzyme (Cellulase Plus Dyadic®) on gas production after 3, 6, and 9 h, ammonia-N concentration, dry matter degradability (DMD), total volatile fatty acid (VFA) production, molar proportions of VFA, and acetate/propionate ratio (Ac/Pr) after 9 h *in vitro* fermentation of samples (400 mg DM) of *Pennisetum purpureum* clon Cuba CT-115 hay (tropical grass hay), maize stover and rice straw in batch cultures of ruminal microorganisms (n=4)

Substrate	TL2 dose[4]	Gas (mL)			NH_3-N	DMD	VFA	Molar Proportion (mol/100 mol)[1]				Ac: Pr
		3 h	6 h	9 h	(mg/L)	(%)	(μmol)	Ac	Pr	But	Others	(mol/mol)
Tropical	C	21.9	31.3	37.6	203	16.2	422	72.8	20.9	4.44	1.80	3.60
grass hay	D1	22.3	33.7*	40.3	195	18.9	464	72.3	21.8	4.77	1.10	3.44
	D2	22.7	34.7*	41.1	191†	20.5*	512*	71.5	22.4	5.05	1.10	3.30
	D3	22.7	35.2*	42.5	190*	19.6*	571*	70.7	22.9*	5.28†	1.06	3.17
	D4	23.7†	41.4*	50.9	188*	21.9*	779*	66.9*	25.5*	6.02*	1.51	2.69*
Maize stover	C	21.6	27.3	30.9	205	6.8	230	76.4	20.0	2.35	1.25	3.93
	D1	22.3	30.1*	35.0	194	10.6*	349*	75.0	19.9	4.21*	0.93	4.00
	D2	23.7*	32.5*	37.3	194	12.4*	376*	74.0	20.5	4.79*	0.75	3.75
	D3	23.5*	32.3*	38.0	191*	12.1*	444*	71.9*	22.2*	5.15*	0.75	3.38*
	D4	25.3*	39.4*	46.9	191*	13.3*	627*	66.1*	25.8*	6.30*	1.83	2.65*
Rice straw	C	18.5	25.5	28.2	183	10.4	157	70.2	21.8	2.76	5.27	3.49
	D1	19.0	25.3	29.4	174	10.9	225*	74.8*	17.3*	3.02	4.87	4.61*
	D2	18.1	25.2	29.7	178	14.2*	219*	73.8*	19.2*	3.10	3.84	4.03*
	D3	19.2	26.1*	30.7	171	15.0*	284*	73.3*	18.9*	5.08*	2.76	4.09*
	D4	20.5*	31.8*	38.1	180	15.6*	554*	68.2†	23.3†	5.94*	2.48	3.06*
SEM[2]		0.70	0.71	0.73	4.6	1.22	22.4	1.06	0.72	0.326	0.975	0.193
P values[3]												
SUB		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.032	<0.001	<0.001	<0.001	<0.001
SUB x ENZ		0.704	0.012	0.017	0.932	0.688	0.311	0.016	0.008	0.002	0.756	0.040
L		0.968	0.245	0.582	0.006	0.054	0.649	0.021	0.002	0.003	0.235	0.009
Q		0.101	<0.001	<0.001	0.247	<0.001	<0.001	0.126	0.188	0.007	0.790	0.561
C		<0.001	<0.001	<0.001	0.090	<0.001	<0.001	<0.001	<0.001	<0.001	0.277	<0.001

*For each substrate and variable, means differ from control ($P<0.05$). †For each substrate and variable, means differ from control ($P<0.10$)

[1] Ac: acetate; Pr: propionate; But: butyrate; Others: sum of isobutyrate, isovalerate and valerate

[2] Standard error of the mean

[3] SUB: substrate; ENZ: enzyme; L, Q and C: linear, quadratic and cubic effect of ENZ dose, respectively

[4] CON: control; D1, D2, D3 and D4 correspond to 1/200, 1/100, 1/50 and 1/10 dilutions of commercial enzymes, respectively. See text for description of enzymes.

for VFA production ($P<0.001$) with TL1. The lower dose of TL1 (D1) increased VFA production by 25, 67 and 30% for grass hay, maize stover and rice straw, respectively, and the higher dose (D4) increased VFA production by 91, 237 and 284% for the same forages. Significant ENZ x forage interactions ($P=0.40$ to <0.001) were also detected in molar proportions of acetate and propionate and Ac: Pr ratio with all tested ENZ. Treatment of rice straw with PEN did not affect ($P>0.05$) either proportions of acetate and propionate or Ac: Pr ratio only, but PEN at D4 decreased ($P<0.05$) acetate proportion and Ac: Pr ratio and increased ($P<0.05$) propionate proportion for maize stover. The TL1 treatment decreased ($P<0.05$) acetate proportion and Ac: Pr ratio and increased ($P<0.05$) propionate proportion for grass hay at D3 and D4 doses and for maize stover at all tested doses, but its effects for rice straw varied with the dose. Whereas treatment of rice straw with TL1 at D1, D2 and D3 increased ($P<0.05$) acetate proportion and Ac: Pr ratio and decreased ($P<0.05$) propionate proportion, D4 dose resulted in decreased ($P<0.05$) acetate proportion and Ac: Pr ratio and increased ($P<0.05$) proportion of propionate. Similar results were observed for TL2, as D4 dose decreased ($P<0.05$) acetate proportion and Ac: Pr ratio and increased ($P<0.05$) propionate proportion for all forages (a trend for rice straw), whereas applied at rice straw at D1, D2 and D3 doses increased ($P<0.05$) acetate proportion and Ac: Pr ratio and decreased ($P<0.05$) propionate proportion.

DISCUSSION

The gas production technique was chosen for this study because it is a simple screening tool to evaluate substrate degradation, and therefore it has been proposed as a powerful screening tool for selecting ENZ additives that improve forage utilization (Beauchemin and Holtshausen, 2010). The lack of effect of TL1 and TL2 on asymptotic gas production and OMD_{120} supports the previous idea that some enzymes increase the rate rather than the extent of feed degradation in the rumen (Beauchemin *et al.*, 2003). In contrast, other studies (Giraldo *et al.,* 2008ab; Kruger *et al.,* 2008ab) have reported that enzymes can also increase the extent of digestion. In our study, some ENZ treatments increased AFR and OMED and decreased $t_{1/2}$, thus indicating a stimulation of forage fermentation which resulted in increased OM digestion. All ENZ were effective at D4, but the efficacy of D1, D2 and D3 was highly variable depending on the ENZ and the forage tested, supporting observations from previous studies (Giraldo *et al.*, 2008b; Krueger *et al.*, 2008; Ranilla *et al.*, 2008). Thus, all doses of PEN were effective with grass hay, but only D4 had a positive effect with maize stover and rice straw. Similarly, TL2 was effective at all doses for grass hay, but only at D3 and D4 for maize stover and at D2, D3 and D4 for a rice straw. In contrast, TL1 was only effective at D4 with all forages. These results indicate the importance of testing fibrolytic ENZ on different feeds.

Based on the positive results observed in the gas production trial, we decided to analyse the effects of ENZ treatments on forage composition and ruminal fermentation. The efficacy of fibrolytic ENZ to reduce NDF or ADF content of forages and forage-

based diets has been shown in previous research (Giraldo *et al.*, 2007ab, 2008a; Dean *et al.*, 2008; Krueger *et al.*, 2008). In our study, highly significant ENZ x forage interactions were observed for the three tested ENZ, indicating that effectiveness of each ENZ varied with the forage to which it was applied. The positive effects of ENZ on fibre composition of forages were, in general, correlated with their effects on ruminal fermentation, but some results indicate that the reduction of fibre content alone cannot explain the ENZ effects. Whereas only D3 and D4 doses of TL1 decreased ADF content of maize stover and rice straw, all tested doses increased or tended to increase DMD and VFA production and modified molar proportions of the main VFA with both forages. Similarly, NDF and ADF content of rice straw was decreased by all doses of TL2, but only by TL1 at D3 and D4; nevertheless, effects of both ENZ on rice straw fermentation were rather similar. These results indicate that other mechanisms of action of ENZ than direct effects on cell wall are probably involved in the observed response.

Previous research (Wang *et al.*, 2001; Giraldo *et al.*, 2007ab) showed clearly that treatment of forage or forage-based diets with fibrolytic ENZ stimulated the initial phases of microbial colonization in the rumen, and this is recognized as one mode of action of ENZ products (Beauchemin and Holtshausen, 2010). Because some studies (Nsereko *et al.*, 2000; Giraldo *et al.*, 2008ab; Ranilla *et al.*, 2008) have shown that the effects of enzymes became less marked as incubation time progressed, we decided to conduct 9 h incubations to assess the effects of ENZ shortly after incubation. Low-quality forages are usually characterized by low intakes, as their slow digestion rates result in long retention times of digesta in the rumen. These problems can be overcome by applying treatments such as enzymes that stimulate the initial phases of forage degradation and thus increase rumen turnover of digesta and forage intake (Leng, 1990). The increased gas production observed at 6 and 9 h of incubation for some ENZ treatments indicates a stimulation of fermentation, as gas production is closely correlated with the amount of organic matter fermented. The stimulation of fermentation was also reflected in the augmented VFA production, which was specially marked for D4 of all ENZ, as it was increased by 1.8, 3.0 and 2.7 times (means across forages) by PEN, TL1 and TL2, respectively. Lower doses of TL1 and TL2 were also effective, as D1 increased VFA production by 1.7 and 1.1 for TL1 and TL2, respectively; D2 by 1.6 and 1.4, and D3 by 1.9 and 1.7 (means across forages). Additionally, a shift in VFA profile was observed for some doses of ENZ, although the effects varied with the forage incubated. For grass hay and maize stover, TL1 and TL2 increased propionate proportions and decreased Ac: Pr ratio. Similar changes have been reported by others (Yang *et al.*, 2002; Krueger and Adesogan, 2008), indicating that fibrolytic ENZ can make the fermentation more gluconeogenic, and hence improve the energetic efficiency of the fermentation (Krueger and Adesogan, 2008). In contrast, for rice straw TL1 and TL2 at D1, D2 and D3 decreased propionate proportions and increased Ac: Pr ratio, but at D4 both ENZ increased propionate proportions and decreased Ac: Pr ratio. This illustrates the variable effects of ENZ on the same substrate when applied at different

doses. High doses of ENZ have been reported to reduce forage fermentation (Beauchemin *et al.*, 2003), but in our study this was not observed as indicated by VFA production and DMD values.

In this study we decide to use the same dilutions of all ENZ, which resulted in similar amounts of ENZ activity applied to forages for the three tested products. Nevertheless, TL1 and TL2 were more effective at stimulating ruminal fermentation compared with PEN. It should be noted that that ENZ activities were measured on pure substrates that do not represent the complexity of plant cell wall, and that these assays were based on the initial rate of reaction with the substrate, which is not related to overall enzyme persistency (Beauchemin *et al.*, 2003). Moreover, it is possible that some of the tested products had additional ENZ activities, such as esterases or proteases, which were not analysed for in our study, thus contributing to stimulate *in vitro* rumen fermentation.

CONCLUSIONS

The results of this research show that treatment of low-quality forages with commercial ENZ reduced their fiber contents and improved their ruminal degradation at short-incubation times, as indicated by the increased VFA production and forage degradability. The reduction of fibre content of forages by ENZ treatment did not fully explain the positive effects of ENZ on ruminal fermentation indicating that other mechanisms of action are involved. The ENZ dose necessary to achieve a positive effect varied with the commercial product and the tested forage, which underlines the importance of testing ENZ products with a range of forages before using them in practical feeding.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial support received from the MICINN (Project AGL2008-04707-C02-02), MAE-AECID (Project A/4951/06) and IAEA (Technical Contract No. 16300). A. Díaz and C. Saro gratefully acknowledge receipt of grants from the AECID and Spanish M.E.C. (AP2006-03049), respectively.

REFERENCES

AOAC. 1999. *Official Methods of Analysis*, 16th ed. AOAC International, Gaithersburg, MD, USA.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science*, 81: E37-E47.

Beauchemin, K.A. and Holtshausen, L. 2010. Developments in enzyme usage in ruminants. In: *Enzymes in Farm Animal Nutrition*, 2nd ed. (Eds. M.R. Bedford and G.G. Partridge), CABI, Oxford, United Kingdom, pp. 206-230.

Carro, M.D. and Miller, E.L. 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semicontinuous culture system (RUSITEC). *British Journal of Nutrition*, 82: 149-157.

Carro, M.D., Ranilla, M.J. and Tejido, M.L. 2005. Using the *in vitro* gas production technique to test feed additives: effects of correcting values for different blanks. *Animal Feed Science and Technology*, 123: 173-184.

Dean, D.B., Adesogan, A.T., Krueger, N.A. and Littell, R.C. 2008. Effects of treatment with ammonia or fibrolytic enzymes on chemical composition and ruminal degradability of hays produced from tropical grasses. *Animal Feed Science and Technology*, 145: 68-83.

Eun, J.S. and Beauchemin K.A. 2007. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. *Animal Feed Science and Technology*, 132: 298-315.

Giraldo, L.A., Ranilla, M.J., Tejido, M.L. and Carro, M.D. 2007a. Influence of exogenous fibrolytic enzyme and fumarate on methane production, microbial growth and fermentation in Rusitec fermenters. *British Journal of Nutrition*, 98: 753-761.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2007b. Effects of exogenous cellulase supplementation on microbial growth and ruminal fermentation of a high-forage diet in Rusitec fermenters. *Journal of Animal Science*, 85, 1962-1970.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J., Ramos, S. and Carro, M.D. 2008a. Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay based diet. *Journal of Animal Science*, 86: 1617-1623.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2008b. Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage: concentrate ratios. *Animal Feed Science and Technology*, 141: 306-325.

Goering, M.K. and Van Soest, P.J. 1970. Forage Fiber Analysis (apparatus, reagents, procedures and some applications). Agricultural Handbook, no 379. Agricultural Research Services, USDA. Washington DC.

Krueger, N.A. and Adesogan, A.T. 2008. Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. *Animal Feed Science and Technology*, 145: 84-94.

Krueger, N.A., Adesogan, A.T., Staples, C.R., Krueger, W.K., Deana, D.B. and Littell, R.C. 2008. The potential to increase digestibility of tropical grasses with a fungal, ferulic acid esterase enzyme preparation. *Animal Feed Science and Technology*, 145: 95-108.

Leng, R.A. 1990. Factors affecting the utilization of 'poor quality' forages by ruminants particularly under tropical conditions. *Nutrition Research Rev*iews, 3: 277-303.

McAllister, T.A., Hristov, A.N., Beauchemin, K.A., Rode, L.M. and Cheng, K.J. 2000. Enzymes in ruminant diets. In: *Enzymes in Farm Animal Nutrition* (Eds. M.R. Bedford and G.C. Partridge), CABI, Oxford, United Kingdom, pp. 273-298.

NRC. 2007. *Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids*. National Research Council, The National Academies Press, Washington DC, USA.

Nsereko, V.L., Morgavi, D.P., Rode, L.M., Beauchemin, K.A. and McAllister, T.A. 2000. Effects of fungal enzyme preparations on hydrolysis and subsequent degradation of alfalfa hay fiber by mixed rumen micro-organisms *in vitro*. *Animal Feed Science and Technology*, 88: 153-170.

Ranilla, M.J., López, S., Giráldez, F.J., Valdés, C. and Carro, M.D. 1998. Comparative digestibility and digesta flow kinetics in two breeds of sheep. *Animal Science*, 66: 389-396.

Ranilla, M.J., Tejido, M.L., Giraldo, L.A., Tricçarico, J.M. and Carro, M.D. 2008. Effects of an exogenous fibrolytic enzyme preparation on *in vitro* ruminal fermentation of three forages and their isolated cell walls. *Animal Feed Science and Technology*, 145: 109-121.

SAS Institute Inc, 2012. SAS/STAT® 12.2 User's guide. SAS Inst Inc, Cary, NC, USA.

Wang, Y., McAllister, T.A., Rode, L.M., Beauchemin, K.A., Morgavi, D.P., Nsereko, V.L., Iwaasa, A.D. and Yang, W. 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the Rumen Simulation Technique (Rusitec). *British Journal of Nutrition,* 85: 325-332.

Yang, W.Z., Beauchemin, K.A. and Vedres, D.D. 2002. Effects of pH and fibrolytic enzymes on digestibility, bacterial protein synthesis, and fermentation in continuous culture. *Animal Feed Science and Technology*, 102: 137-150.

Animal Nutrition and Feed Technology (2013) 13: 477-487

Synergistic Effect of Cellulase and Xylanase on *In Vitro* Rumen Fermentation and Microbial Population with Rice Straw as Substrate[#]

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ABSTRACT

Mao, H.L., Wu, C.H., Wang, J.K. and Liu, J.X. 2013. Synergistic effect of cellulase and xylanase on in vitro rumen fermentation and microbial population with rice straw as substrate. Animal Nutrition and Feed Technology, 13: 477-487.

The *in vitro* gas test was conducted to investigate the effects of cellulase (CEL) and xylanase (XYL) on *in vitro* rumen fermentation and microbial population with rice straw as substrate. Three levels of CEL (0, 7.5 and 15 U/g of DM) and XYL (0, 15 and 30 U/g of DM) were tested in a 3×3 factorial arrangement. Addition of CEL and XYL could affect the gas production parameters. Methane emission was increased linearly by CEL addition ($P<0.01$), while the methane production was greater when XYL added at the intermediate dose (quadratic, $P<0.01$). Total volatile fatty acids (CEL, quadratic, $P<0.01$; XYL, linear and quadratic, $P<0.05$) were enhanced by either CEL or XYL addition. Ammonia nitrogen was reduced by CEL (linear and quadratic, $P<0.05$), while microbial protein was enhanced by addition of CEL (quadratic, $P<0.01$) and XYL (linear and quadratic, $P<0.01$). *In vitro* degradability of DM and NDF were increased by addition of CEL (quadratic, $P<0.05$) or XYL (quadratic, $P<0.01$). Addition of CEL increased the copy number of total bacteria (linear, $P<0.01$) and *Fibrobacter succinogens* (linear and quadratic, $P<0.05$). The outcome of this research indicated that the application of CEL and XYL could improve rumen fermentation, increase rice straw digestion and affect the rumen microbial population. Combination of CEL and XYL at the middle level is more effective than a single CEL or XYL.

Key words: Cellulase, Microbial population, Rice straw, Rumen fermentation, Xylanase

INTRODUCTION

In rice-producing countries in the tropics, rice straw constitutes an important source of roughage for ruminants. It has been well recognized that rice straw is nutritionally poor, because of its low content of available energy and nitrogen, high fiber content, and poor digestibility and efficiency of utilization by ruminants (Wang *et al.*, 2007).

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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Use of exogenous enzymes in ruminant feeds has improved forage utilization and productivity, and reduced nutrient excretion. However, the effects of enzymes are influenced by factors such as type and dose of enzyme, type of diet fed to animals, enzyme application method, and even the level of animal productivity (Beauchemin *et al.*, 2003). Regarding the factors related to the diet, the effectiveness of fibrolytic enzymes has been shown to vary with forage (Colombatto *et al.*, 2003a; Wallace *et al.*, 2001), enzyme application method (Giraldo *et al.*, 2004) and the component of the diet to which the enzyme is added (Beauchemin *et al.*, 2003). The focus of most enzyme-related research for ruminants has been on plant cell wall degrading enzymes, cellulases and hemicellulases, to degrade cellulose and hemicellulose, respectively (Liu and Orskov, 2000; Eun and Beauchemin, 2007a). In our previous study, it was found that addition of single exogenous fibrolytic enzyme increased degradation of corn silage (Chen *et al.*, 2012). Because cellulases and hemicellulases act synergistically to hydrolyze plant cell wall, enzymes with cellulase and hemicellulase activities may be more efficient in degrading forage compared with enzymes with either single activity. Thus, the objective of this study was to evaluate the effects of cellulase (CEL), xylanase (XYL) and their combination on *in vitro* rumen fermentation and microbial population with rice straw as substrate.

MATERIALS AND METHODS

Forage and enzyme products

The rice straw was grounded by a miller (DFT-50, Lin-tai Machinery Co., Ltd) to pass a 2 mm screen. Chemical composition (DM %) of rice straw was as below: 6.78 crude protein, 13.1 acid detergent fiber, and 71.6 neutral detergent fiber (NDF). Cellulase and XYL products were obtained from Sunhy Animal Pharmacy Co., Ltd (Wuhan, China) (Table 1). Cellulase was solid, while XYL was liquid.

Table 1. Protein concentration and enzymatic activities of the enzymes products

Enzyme[†]	Protein concentration[‡] mg/g	Enzyme activity[§] U/g		
		Xylanase	Endoglucanase	Exoglucanase
CEL	18.1	1 041	867	17
XYL	8.2	29 862	1	1

[†]CEL=Cellulase; XYL=Xylanase; [‡]Protein content was expressed as mg of protein g-1 the enzyme products; [§]Enzyme activities were expressed as μmol of sugar released min^{-1} g^{-1} or mL^{-1} of the enzyme products at 39°C, pH 6.6.

Experimental design

An experiment was carried out according to a 3×3 factorial design with CEL and XYL as main effects. The addition levels of CEL were 0, 7.5 and 15 endoglucanase units/g of substrate DM, and the XYL addition levels were 0, 15 and 30 xylanase units/g of substrate DM.

In vitro fermentation

An *in vitro* gas production was determined with semi- automated Reading Pressure Technique (RPT, Mauricio *et al.*, 1999). Rumen fluid was collected from three donor sheep fed a mixed diet of alfalfa hay and concentrate mixture (50: 50, w/w) fed twice daily. Ingredients (% DM) of concentrate mixture included corn (50), wheat bran (15), soybean meal (15), rapeseed meal (13), dicalcium phosphate (2.0), salt (1.5), calcium carbonate (1.5) and vitamin-trace mineral premix (2), and were formulated to provide (per kg of DM) 1,200,000 IU of vitamin A, 280,000 IU of vitamin D, 5000 mg of vitamin E, 14,000 mg of Zn, 100 mg of Se, 200 mg of I, 3,000 mg of Fe, 60 mg of Co, 3,500 mg of Mn, and 3,000 mg of Cu.

Rumen fluid (10 ml) was injected into 120 ml bottles containing 40 ml of buffered medium (Theodorou *et al.*, 1994) and 1.0 g rice straw at 39°C under anaerobic conditions.

Chemical analyses

The amount of protein present in the enzyme products was determined using the Super-Bradford protein determination kit (Cat. No. CW-0013). The enzyme products were analysed for their endoglucanase, exoglucanase and xylanase activities according to the procedures reported by Wood and Bhat (1988) and Bailey *et al.* (1992). The assay conditions were 39°C and the pH 6.6 to reflect ruminal conditions.

After incubation at 39°C for 24 h, rumen fluid was sampled to determine pH, ammonia nitrogen, volatile fatty acid (VFA), and microbial crude protein (MCP). For determination of the absolute quantitative of total bacterial, *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens*, two aliquots of fluid from each bottle were sampled under oxygen-free CO_2, and stored at -80°C.

Fermentation parameters

The pH of rumen liquor was determined by using a pH meter (Model PB-20, Sartorius). Ammonia-N concentration was determined by a spectrometer (Model 721) using colorimetry with the NH_4Cl solution as a standard (Feng *et al*, 1993). For determination of VFAs, 2 ml sample of the fermentation medium was placed in centrifugal tubes, mixed uniformly with 0.5 ml of 25% ortho-phosphoric acid, and then centrifuged at 10000 rpm for 10 min. The supernatant was analysed using gas chromatography (GC-8A, Shimadzu). The temperature of the injector/detector and the column were 260°C and 220°C, respectively. Concentrations of the MCP estimated using a purine method Makkar and Becker (1999). Methane concentration was also analysed using gas chromatography (GC-8A, Shimadzu). The temperatures of the injector/detector and the column were 130°C and 80°C, respectively (Hu *et al.*, 2005).

Total DNA extraction and real-time quantitative PCR

Total DNA was extracted from rumen fluid by bead-beating method as described

by Zhang *et al*. (2008). The amplifying primer sets of total bacterial, *F. succinogenes, R. albus and R. flavefaciens* are listed in Table 2. The absolute quantitative real-time PCR was performed using the ABI 7500 real time PCR system (Applied Biosystems, USA) as described by Tajima *et al*. (2001) and Chen *et al*. (2008), with fluorescence detection of SYBR Green dye.

Table 2. PCR primers for real-time PCR assay

Target Species	Forward	Primer sequence
Total bacteria[1]	F	CGGCAACGAGCGCAACCC
	R	CCATTGTAGCACGTGTGTAGCC
R. flavefaciens[1]	F	CGAACGGAGATAATTTGAGTTTACTTAGG
	R	CGGTCTCTGTATGTTATGAGGTATTACC
R. albus[2]	F	CCCTAAAAGCAGTCTTAGTTCG
	R	CCTCCTTGCGGTTAGAACA
F. succinogenes[1]	F	GTTCGGAATTACTGGGCGTAAA
	R	CGCCTGCCCCTGAACTATC

[1]Cited from Denman and McSweeney (2006); [2]Cited from Koike and Kobayashi (2001).

Calculation and statistical analyses

The standard curves of total bacterial, *F. Succinogenes, R. albus* and *R. flavefaciens* were as follows: Total bacterial $Y=-3.2062X + 37.315$ ($R^2=0.9941$, $E=105.07\%$); *F. succinogenes* $Y=-3.3615X + 38.015$ ($R^{2=}0.9986$, $E=98.38\%$); *R. albus* $Y=-3.4775X + 42.038$ ($R^2=0.9937$, $E=93.89\%$); *R. flavefaciens* $Y=-3.2483X + 40.534$ ($R^2=0.9945$, $E=103.17\%$), where X is logarithm of copy numbers, Y is the threshold cycle, E is amplification efficiency.

To describe the dynamics of *in vitro* gas production over time, the following Gompertz function was chosen (Schofield *et al.*, 1994): $GP=A\exp\{-\exp[1 + Be (LAG - t)/A]\}$, where GP is the cumulative gas production (mL) at time point t, A is the theoretical maximum of gas production (mL), B is the maximum rate of gas production (mL/h), e is the Euler constant (base of the natural logarithm), and LAG is the lag time.

All the statistical analyses were conducted using the GLM procedures (SAS, 1999). Data were analysed as two-way analysis of variance. Orthogonal polynomial contrast was used to examine the responses to increasing levels of CEL and XYL.

RESULTS

Effects of addition of CEL and XYL on *in vitro* gas production parameters and methane emission of rice straw are showed in Table 3. The cumulative gas production (CEL, quadratic, $P<0.01$; XYL, quadratic, $P<0.01$), A (CEL, quadratic, $P<0.05$; XYL, quadratic, $P<0.01$), and B (CEL, quadratic, $P<0.01$; XYL, quadratic, $P<0.01$) were increased as the increasing levels of CEL and XYL. The CEL×XYL interactions were also observed on these three parameters. The LAG decreased linearly

in response to CEL addition ($P<0.01$), but was not affected by XYL ($P>0.05$). Methane emission was increased for CEL and XYL addition (CEL, linear, $P<0.01$; XYL, quadratic, $P<0.01$).

Table 3. Effects of cellulase and xylanase addition on *in vitro* gas production (GP) and methane emission with rice straw as a substrate

Cellulase	Xylanese	GP† at 48 h				Methane
U/g of DM	U/g of DM	GP, mL	A, mL	B, mL/h	LAG, h	mmol/L
0	0	98.8	102	3.77	5.89	2.10
0	15	111	115	4.21	4.44	2.67
0	30	104	106	3.74	4.37	2.53
7.5	0	106	109	3.92	3.03	2.86
7.5	15	106	110	3.85	3.63	2.82
7.5	30	101	104	3.81	4.01	2.72
15	0	94.7	100	3.26	3.61	2.61
15	15	105	109	3.80	4.10	2.98
15	30	98.7	103	3.53	3.47	2.61
Pooled SEM		1.46	1.93	0.074	0.476	0.085
Effect						
Cellulase		**	*	**	*	**
Linear		ns	ns	ns	**	**
Quadratic		**	*	**	ns	ns
Xylanase		**	**	**	ns	**
Linear		ns	ns	ns	ns	ns
Quadratic		**	**	**	ns	**
CEL*XYL		**	*	**	ns	**

** $P<0.01$; * $P<0.05$; ns=not significant

†GP=the cumulative gas production; A=the theoretical maximum of gas production; B=the rate of gas production; and LAG=the lag time.

In vitro degradability of DM (DMD) (CEL and XYL, quadratic, $P<0.05$) and NDF (NDFD) (CEL and XYL, quadratic, $P<0.0$) were increased by addition of CEL and XYL, and greater values for DMD and NDFD were observed at middle adding levels of CEL and XYL (Table 4). There was interaction of CEL and XYL on the NDFD.

The fermentation parameters of rice straw affected by CEL and XYL are presented in Table 5. Total VFA were enhanced by either CEL (quadratic, $P<0.01$) or XYL (linear and quadratic, $P<0.05$). Addition of CEL increased acetate but decreased propionate proportion (linear and quadratic, $P<0.01$), thus the ratio of acetate to propionate was increased (linear and quadratic, $P<0.01$). Ammonia nitrogen was reduced by CEL (linear and quadratic, $P<0.05$), while MCP was enhanced by addition of CEL (quadratic, $P<0.01$), XYL (linear and quadratic, $P<0.01$) or their combination ($P<0.01$). The CEL×XYL interactions were observed on total VFA and MCP.

Table 4. Effect of cellulase and xylanase addition on DM and NDF degradation of rice straw

Cellulase U/g of DM	Xylanase U/g of DM	Degradation ratio, %	
		DM	NDF
0	0	49.1	55.7
0	15	52.3	60.6
0	30	47.8	56.6
7.5	0	46.2	53.9
7.5	15	56.0	62.9
7.5	30	49.9	55.8
15	0	55.6	62.5
15	15	53.5	60.6
15	30	55.1	62.1
Pooled SEM		1.59	0.86
Effect			
Cellulase		*	**
Linear		ns	ns
Quadratic		*	**
Xylanase		*	**
Linear		ns	ns
Quadratic		*	**
CEL*XYL		ns	*

** P<0.01; * P<0.05; ns=not significant.

Table 5. Effects of cellulase and xylanase addition on *in vitro* fermentation parameters of rice straw

Cellulase U/g of DM	Xylanase U/g of DM	VFA† mmol/L	Molar proportion (mol/100mol)			A: P	NH_3-N mg/L	MCP‡ mg/ml
			Acetate	Propionate	Butyrate			
0	0	76.2	64.2	20.7	8.21	3.11	109	7.0
0	15	79.8	64.4	21.2	7.92	3.04	112	9.0
0	30	77.1	64.7	20.6	7.92	3.14	110	8.6
7.5	0	79.6	66.7	18.2	8.51	3.67	107	8.3
7.5	15	75.9	65.9	18.8	8.52	3.52	106	9.3
7.5	30	74.6	65.9	19.4	7.75	3.39	105	7.4
15	0	72.6	66.8	17.8	8.63	3.76	107	7.5
15	15	73.8	66.6	18.1	8.37	3.68	102	7.8
15	30	70.9	66.2	18.5	8.32	3.58	105	8.4
Pooled SEM		0.77	0.38	0.50	0.177	0.111	2.2	0.18
Effect								
Cellulase		**	**	**	*	**	*	*
Linear		ns	**	**	ns	**	*	ns
Quadratic		**	**	**	*	**	*	**
Xylanase		*	ns	ns	*	ns	ns	**
Linear		*	ns	ns	*	ns	ns	**
Quadratic		*	ns	ns	ns	ns	ns	**
CEL*XYL		**	ns	ns	ns	ns	ns	**

** P<0.01; * P<0.05; ns=not significant; †VFA=volatile fatty acid; ‡MCP=microbial crude protein.

Results for rumen microbial populations are shown in Table 6. Addition of CEL increased the copy number of total bacteria (linear, $P<0.01$) and *F. succinogens* (linear and quadratic, $P<0.05$), and the greater values were observed when CEL and XYL added at the middle levels. The *R. albus* and *R. flavefaciens* population were not affected by CEL or XYL addition ($P>0.05$).

Table 6. Effects of cellulase and xylanase addition on rumen microbial populations incubated with rice straw

Cellulase U/g of DM	Xylanase U/g of DM	Copy number			
		Total bacteria $\times10^7$	*R.albus* $\times10^4$	*R.flavefaciens* $\times10^3$	*F.succinogens* $\times10^6$
0	0	1.66	2.62	1.50	2.55
0	15	1.59	3.20	2.12	2.34
0	30	1.71	2.63	1.13	2.72
7.5	0	1.86	3.36	3.34	3.83
7.5	15	1.87	3.99	6.23	4.02
7.5	30	1.82	3.04	1.53	2.44
15	0	1.96	3.84	1.40	2.30
15	15	1.77	3.70	1.40	2.33
15	30	1.57	2.17	1.44	1.35
Pooled SEM		0.053	0.632	1.446	0.480
Effect					
Cellulase		**	ns	ns	**
Linear		**	ns	ns	*
Quadratic		ns	ns	ns	*
Xylanase		*	ns	ns	ns
Linear		*	ns	ns	ns
Quadratic		ns	ns	ns	ns
CEL*XYL		*	ns	ns	ns

** $P<0.01$; * $P<0.05$; ns = not significant.

DISCUSSION

As compared to Eun and Beauchemin (2003), the cellulase activity was lower but the xylanase activity was higher. The activities of enzyme used in this study were measured at pH 6.6, while it was 6.0 in Eun and Beauchemin (2003). Enzyme activities were determined at physiological conditions, which generally differed from the optimal conditions. And the conditions closely resembled the conditions under which the enzymes are expected to act (Colombatto *et al.*, 2003c).

Cellulase and other commercial fibrolytic enzymes can increase the cumulative GP and rate of *in vitro* fermentation of grass (Wallace *et al.*, 2000). Increasing level of fibrolytic enzymes increased rate and GP using rice straw as a substrate (Tang *et al.*, 2008). However, some studies reported contrary results that the fibrolytic enzyme did not affect the rate of gas production (Kung *et al.*, 2002; Eun and Beauchemin, 2007b). In the present study, addition of CEL and XYL increased the GP parameters. The effects of fibrolytic enzymes addition may be influenced by diet composition, type of

enzyme used, enzyme addition level, enzyme stability and method of application (Yang *et al.*, 2000).

Feng *et al.* (1996) found that *in vitro* or *in situ* DM and NDF disappearance of cool-season grass could be increased by adding fibrolytic enzyme directly before incubation. Feed enzyme products increased DM and fiber degradation of alfalfa hay, but they had no effect on DM degradation while increased degradation of NDF of corn silage (Eun and Beauchemin, 2007). A possible mode of action of fibrolytic enzymes was that pretreatment of feed with enzymes could release the reducing sugar (Nsereko *et al.*, 2000), while reducing sugars and other released products of hydrolysis can enhance ruminal microbial colonization (Giraldo *et al.*, 2007), and consequently reduces particle size of fiber and increases DM digestion (Feng *et al.*, 1996).

Increased gas production and DMD led to an increase in total VFA production (Table 3-5). Enzymes preparations can contain sugars that are fermented by ruminal micro-organisms, thus increasing VFA production (Colombatto *et al.*, 2003b). However, the changes in VFA proportion due to fibrolytic enzyme addition were inconsistent. In the study of Eun and Beauchemin (2007), addition of exogenous fibrolytic enzyme increased the propionate and butyrate proportion and decreased the proportion of acetate. In the current study, XYL did not affect molar portion of VFA except butyrate, but CEL addition resulted in an increase in acetate. Acetate production is associated with the release of H_2, which can be used by methanogens to form methane (Stewart *et al.*, 1997), the increased acetate production is consistent with the greater production of methane observed for CEL treatment. Effects of enzyme addition on VFA composition may depend on the enzyme activities supplied by the exogenous fibrolytic enzyme and their impact on forage fiber degradation and ruminal microbial activities (Eun *et al.*, 2007).

Addition of CEL and XYL increased the production of methane in this study. To our knowledge, only a few studies have investigated the effects of exogenous enzymes on methane emission in the rumen, and the results are inconsistent.) An increase was observed in methane production for exogenous enzyme addition (Dong *et al.*, 1999; Giraldo *et al.,* 2007). In contrast, methane production, expressed as a proportion of total gases, was not affected by enzyme addition in a dual-flow continuous culture system (Colombatto *et al.*, 2003a) or in steers fed a barley silage based diet (McGinn *et al.*, 2004).

Enzyme addition tended to increase the total viable bacterial count, but had no effect on the cellulolytic bacteria populations (Colombatto *et al.*, 2003a). In the study of Nsereko *et al.* (2002), inclusion of an exogenous fibrolytic enzyme preparation in dairy cow diets increased the numbers of rumen bacteria that utilize hemicellulose and secondary products of cellulose digestion, but did not affect the numbers of cellulolytic bacteria. In the present study, CEL addition resulted in an increase in total bacteria and *F. succinogenes* numbers, but had no effect on the three cellulolytic bacteria. The use of

enzyme products differing in their biochemical properties makes direct comparisons difficult (Colombatto *et al.*, 2003a).

Most exogenous fibrolytic enzymes contain cellulase and xylanases. Eun *et al.* (2007) found that xylanase play a limited role in the fermentation of alfalfa gay and corn silage. Thus, cellulases and xylanase usually have a synergistic effect on cell wall hydrolyisis (Bhat and Hazlewood, 2001). Hespell and Whitehead (1990) reported that only small amounts of sugars were released when arabinosidase or xylanase were incubated individually with alfalfa cell walls, but sugar release increased 5- to 10- fold when the enzymes were used together. In our study, combination of CEL and XYL at the middle level was more effective as compared to other treatments. In the work of Eun and Beauchemin (2007), it was observed that combination treatments resulted in no further beneficial effects on the degradation of either forage when endoglucanase and xylanase from single-activity enzyme products were combined in a 1:1 ratio. There might be an optimal ratio between the major enzymatic activities to achieve further improvement of degradation with combination treatments (Eun and Beauchemin, 2007). For alfalfa hay and corn silage, the optimal ratio of endoglucanase and xylanase appeared to be＞0.4:1 (Eun *et al.*, 2007). In this study, the combined CEL (7.5U/g of DM) with XYL (15U/g of DM) was more effective, and the optimal ratio of endoglucanase to xylanase was at 0.5: 1.

CONCLUSION

Addition of CEL and XYL could improve rumen fermentation, increase rice straw DMD and NDFD and enhance the rumen bacterial numbers. Combination of CEL (7.5U/g of DM) and XYL (15U/g of DM) was more effective than a single CEL or XYL.

ACKNOWLEDGEMENT

Supported in part by grants from Joint FAO/ IAEA Division, IAEA, Vienna (Austria), Research Contract No. 16327 within the D3.10.27 Coordinated Research Project.

REFERENCES

Bailey, M.J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology,* 23: 257-270.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science,* 81 (E. Suppl. 2): E37-47.

Bhat, M.K. and Hazlewood, G.P. 2001. Enzymology and other characteristics of cellulases and xylanases. In: *Enzymes in Farm Animal Nutrition*. CABI Publishing, Wallingford, Oxon, UK, pp. 11-60.

Chen, X., Wang, J.K., Mao, H.L., Wu, C.H., Wu, Y.M. and Liu, J.X. 2012. Effect of exogenous fibrolytic enzymes on *in vitro* rumen fermentation of corn silage. *Journal of Animal Science,* Vol. 90, Suppl. 3/*Journal of Dairy Science,* Vol. 95, Suppl. 2: 138

Chen, X.L., Wang, J.K. and Liu, J.X. 2008. Development of a real-time absolute quantitative PCR for quantification of cellulolytic bacteria in rumen. *Chinese Journal of Animal Science,* 44: 36-40.

Colombatto, D., Hervás, G., Yang, W.Z. and Beauchemin, K.A. 2003a. Effects of enzyme supplementation of a total mixed ration on microbial fermentation in continuous culture, maintained at high and low pH. *Journal of Animal Science,* 81: 2617-2627.

Colombatto, D., Mould, F.L., Bath, M.K., Morgavi, D.P. and Owen, E. 2003b. Use of fibrolytic enzymes to improve the nutritive value of ruminant diets. A biochemical and *in vitro* rumen degradation assessment. *Animal Feed Science and Technology,* 107: 201-209.

Colombatto, D., Morgavi, D.P., Furtado, A.F. and Beauchemin K.A. 2003c. Screening of exogenous enzymes for ruminant diets: Relationship between biochemical characteristics and *in vitro* ruminal degradation. *Journal of Animal Science,* 81: 2628-2638.

Denman, S.E. and McSweeney, C.S. 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiology Ecology,* 58: 572-582.

Dong, Y., Bae, H.D., McAllister, T.A., Mathison, G.W. and Cheng K.J. 1999. Effects of exogenous fibrolytic enzymes, α-bromoethanesulfonate and monensin on fermentation in a rumen simulation (Rusitec) system. *Canadian Journal of Animal Science,* 79: 491-498.

Eun, J.-S. and Beauchemin, K.A. 2007a. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using *in vitro* fermentation characteristics. *Animal Feed Science and Technology,* 132: 298-315.

Eun, J.-S. and Beauchemin, K.A. 2007b. Enhancing *in vitro* degradation of alfalfa hay and corn silage using feed enzymes. *Journal of Dairy Science,* 90: 2839-2851.

Eun, J.-S., Beauchemin, K.A. and Schulze, H. 2007. Use of exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. *Journal of Dairy Science,* 90: 1440-1451.

Feng, P., Hunt, C.W., Pritchard, G.T. and Julien, W.E. 1996. Effect of enzyme preparations on *in situ* and *in vitro* degradation and *in vitro* digestive characteristics of mature cool-season grass forage in beef steers. *Journal of Animal Science,* 74: 1349-1357.

Feng, Z.C. and Gao M. 1993. The determination of ammonia nitrogen concentration in the rumen fluid by a colorimetric method. *Inner Mongolian Journal of Animal Science and Production*. 4: 40-41. (in Chinese)

Giraldo, L.A., Ranilla, M.J., Tejido, M.L. and Carro, M.D. 2004. Effect of enzyme application method on *in vitro* rumen fermentation of tropical forages. *Journal of Animal and Feed Science,* 13: 63-66.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2007. Effects of exogenous cellulose supplementation on microbial growth and ruminal fermentation of a high-forage diet in Rusitec fermenters. *Journal of Animal Science,* 85: 1962-1970.

Hespell, R.B. and Whitehead, T.R. 1990. Physiology and genetics of xylan degradation by gastrointestinal tract bacteria. *Journal of Dairy Science,* 73: 3013-3022.

Hu, W.L., Liu, J.X., Ye, J.A., Wu, Y.M. and Guo, Y.Q. 2005. Effect of tea saponin on rumen fermentation *in vitro*. *Animal Feed Science and Technology,* 120: 333-339.

Koike, S. and Kobayashi, Y. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefacies*. *FEMS Microbiology Letters,* 204: 361-366.

Kung, L., Cohen, M.A., Rode, L.M. and Treacher, R.J. 2002. The effect of fibrolytic enzymes sprayed onto forages and fed in a total mixed ratio to lactating dairy cows. *Journal of Dairy Science,* 85: 2396-2402.

Liu, J.X. and Ørskov, E.R. 2000. Cellulase treatment of untreated and steam pre-treated rice straw-effects on *in vitro* fermentation characteristics. *Animal Feed Science and Technology,* 88: 189-200.

Makkar, H.P.S. and Becker, K. 1999. Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods. *British Journal of Nutrition.* 81: 107-113.

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S. and Theodorou, M.K. 1999. A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. *Animal Feed Science and Technology,* 79: 321-330.

McAllister, T.A., Hristov, A.N., Beauchemin, K.A., Rode, L.M. and Cheng K.J. 2001. Enzymes in ruminant diets. In: *Enzymes in Farm Animal Nutrition* (Eds. M.R. Bedford and G.G. Partridge), CAB International, U.K, pp. 273-298.

Nsereko, V.L., Beauchemin, K.A., Morgavi, D.P., Rode, L.M., Furtado, A.F., McAllister,T.A., Iwaasa, A.D., Yang, W.Z. and Wang Y. 2002. Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. *Canadian Journal of Microbiology,* 48: 14-20.

SAS. 1999. *SAS Procedures Guide, Version 8.0*. SAS Institute Inc., Cary, NC, USA.

Schofield, P.R., Pitt, E. and Pell, A.N. 1994. Kinetics of fiber digestion from *in vitro* gas production. *Journal of Animal Science,* 72: 2980-2991.

Stewart, C.S., Flint, H.J. and Bryant, M.P. 1997. The rumen bacteria. In: *The Rumen Microbial Ecosystem* (Eds. P.N. Hobson and C.S. Stewart), Chapman and Hall, London, UK, pp. 10-72.

Tajima, K., Aminov, R. and Nagamine T. 2001. Diet-Dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Applied Environmental Microbiology,* 67: 2766-2774.

Tang, S.X., Tayo, G.O., Tan, Z.L., Sun, Z.H., Shen, L.X., Zhou, C.S., Xiao, W.J., Ren, G.P., Han, X.F. and Shen, S.B. 2008. Effects of yeast culture and fibrolytic enzyme supplementation on *in vitro* fermentation characteristics of low-quality cereal straws. *Journal of Animal Science,* 86: 1164-1172.

Wang, J.K., Liu, J.X., Li, J.Y., Wu, Y.M. and Ye, J.A. 2007. Histological and rumen degradation changes of rice straw stem epidermis as influenced by chemical pretreatment. *Animal Feed Science and Technology,* 136: 51-62.

Wallace, R.J., Wallace, S.J.A., McKain, N., Nsereko, V.L. and Hartnell G.F. 2001. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal micro-organisms *in vitro*. *Journal of Animal Science,* 79: 1905-1916.

Wallance, A.J., Wallance, S.J.A., McKain, N., Nsereko, V.L. and Hartnell G.F. 2000. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass by mixed ruminal micro-organisms *in vitro*. *Journal of Animal Science,* 79: 1905-1916.

Wood, T.M. and Bhat, K.M. 1988. Methods for measuring cellulase activities. In: *Methods in Enzymology* (Eds. A. Willis and S.T.K. Wood), Academic Press, pp. 87-112.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science,* 83: 2515-2520.

Zhang, C.M., Guo, Y.Q., Yuan, Z.P., Wu, Y.M., Wang, J.K., Liu, J.X. and Zhu, W.Y. 2008. Effect of octadeca carbon fatty acids on microbial fermentation, methanogenesis and microbial flora *in vitro*. *Animal Feed Science and Technology,* 146: 259-269.

Animal Nutrition and Feed Technology (2013) 13: 489-505

Effects of Exogenous Cellulase Source on *In Vitro* Fermentation Characteristics and Methane Production of Crop Straws and Grasses#

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ABSTRACT

Tang, S.X., Zou, Y., Wang, M., Salem, A.Z.M., Odongo, N.E., Zhou, C.S., Han, X.F., Tan, Z.L., Zhang, M., Fu, Y.F., Huang, S.Q., He, Z.X. and Kang, J.H. 2013. Effects of exogenous cellulase source on in vitro fermentation characteristics and methane production of crop straws and grasses. Animal Nutrition and Feed Technology, 13: 489-505.

In vitro fermentation experiments were conducted to investigate the effects of 3 sources of exogenous cellulase products (EC) at 4 dose rates (DR) (0, 12, 37 and 62 IU/g of DM) on degradation of forage and methane production by mixed rumen micro-organisms of goats. The maximum gas production (*Vf*) of grasses was higher ($P < 0.001$) in *Neocallimastix patriciarum (*NP) group than those in *Trichoderma reesei* (TR) and *Trichoderma longibrachiatum* (TL) groups. Quadratic increases in dry matter degradation (DMD) of forage and neutral detergent fiber (NDFD) of straw were observed for all EC, with optimum DR in the low range. Supplementation of EC originated from TR and NP increased ($P < 0.001$) DMD of forage compared to that from TL. Addition of EC originated from TR and NP also decreased pH value, ammonia nitrogen (NH_3-N) and methane (CH_4) production compared to that from TL. Quadratic decreases in pH value, NH_3-N and CH_4 of forage were noted for EC of TR and NP, and with optimum DR in the low range. For short chain fatty acid, the EC of NP increased total volatile fatty acid (TVFA) and acetate concentration and the ratio of acetate to propionate of forage compared with EC of TL and TR, and with optimum DR in the low to medium range. It was concluded that the source of EC differed in fiber degradation and methane emission, and with optimum DR of TR in the low range (from 12 to 37 U/g DM) in improving fiber degradation and decreasing methane emission.

Key words: Forage, Exogenous cellulase, *In vitro* fermentation, Methane production

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#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

Roughages, especially crop residues constitute the major ingredients of ruminants' diets in developing countries, while the complex network formed by structural carbohydrates and lignin in crop residues limits the digestibility and efficient utilization of forages by ruminants. Many attempts have been made to overcome this limitation, and the use of exogenous enzymes has been received considerable attention for many years (Tang *et al.,* 2008; Malik and Bandla, 2010; Chung *et al.,* 2012). However, the *in vitro* and *in vivo* experimental results have highly variable, the ineffectiveness (Burroughs *et al.,* 1960; Reddish and Kung, 2007) and even negative responses (Sutton *et al.,* 2003) have frequently been observed in previous studies. Meanwhile, they have suggested that the effectiveness of dietary exogenous fibrolytic enzymes supplementation depends not only on the type of ration, but also on activity of enzymes, level and mode of enzyme supplementation, and even on nature and source of enzymes. On the other hand, 15-20% of global methane (CH_4) is produced by ruminants in agriculture production systems (Ding *et al.,* 2012). Methane production from ruminants fed highly fibrous diets is higher than those from animals fed better quality forages. Recent researches have shown that supplementing fiber degrading enzymes in livestock diets may improve feed utilization by enhancing fiber degradation, reduction in methane emission per unit of animal by-products (Nsereko *et al.,* 2002; Reddish and Kung, 2007; Shojaeian and Thakur, 2007). While Chung *et al.* (2012) has reported that CH_4 emissions obviously increased when EC was added in total mixed ration at 0.5 or 1.0 mL/kg DM for dairy cows, whether it was calculated according to per kg of dry matter intake or per kg of milk production. Thus, the objective of present study was conducted to evaluate the effects of dosage and source of EC on the *in vitro* fermentation and CH_4 production from forages.

MATERIALS AND METHODS

This experiment was approved by the Animal Care Committee, Institute of Subtropical Agriculture (ISA), the Chinese Academy of Sciences, Changsha, China.

Crop straws, grasses and enzymes

Three crop straws, i.e., maize stover (Kexiangtian 1), rice straw (Xiang 125s) and wheat straw (Taishan 9818) after grain were harvested, and two grasses, i.e., Guimu 1 at late tillering and alfalfa before flowering were selected as *in vitro* fermentation substrates in this study. They were dried at 65°C for 24 h, and then ground through 1 mm sieve and stored in plastic bag for assay. Maize stover had the following chemical composition (DM basis): 5.3% CP, 63.6% NDF, and 38.6% ADF. The rice straw contained (DM basis): 6.2% CP, 63.2% NDF, and 43.4% ADF. The wheat straw was composed of (on DM basis): 8.4% CP, 68.4% NDF, and 42.6% ADF. The Guimu 1 contained (on DM basis) 8.9% CP, 61.2% NDF and 33.6% ADF, and 10.4% CP, 43.3% NDF, and 30.0% ADF were involved in alfalfa.

Exogenous cellulase (EC) were procured from *Neocallimastix patriciarum* (NP; Hunan Youtell Biochemical., Ltd. Yueyang, China), *Trichoderma Longibrachiatum* (TL; Wuhan Sunhy Biological Co., LTD, Wuhan, China) and *Trichoderma Reesei (*TR; Guangdong VTR Biological Technology Co., Ltd., Zhuhai, China), respectively. All of the EC products were powder form, and are acceptable for use in animal feeds in China.

In vitro gas production and sampling

Culture solutions, i.e., macroelement solution, buffered solution and reducing solution used for *in vitro* fermentation were prepared to form artificial salivary according to the procedures modified by Tang *et al.* (2006). The artificial salivary was anaerobic by pumping carbon dioxide for 2 h.

About 1000±20 mg sample of each straw or grass was accurately weighed into 150 mL fermentation bottles. Each sample was measured in triplicates. Exactly 0.5333 g of NP, 1.2121 g of TL and 0.8889 g of TR enzyme powder were solubilized using 5 mL of water, and 15, 46.25 and 77.5 μl of the diluted enzyme was added to the forage to achieve a DR of 12, 37 and 62 IU of concentrated enzyme product per g of forage DM. Then the samples were stored at room temperature until artificial saliva and rumen fluids were added.

Ruminal fluid was collected before morning feeding from three rumem-cannulated goats fed a corn stover based total mixed ration, and immediately transported to laboratory. Ruminal contents were strained through four layers of cheesecloth under a continuous CO_2 stream. Ten milliliters of ruminal fluids and 90 mL of artificial salivary were introduced to the bottle pre-warmed at 39°C. All bottles were connected with pressure sensors. Then, the bottles were incubated at 39°C.

The pressure in the bottles were recorded at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48 and 72 hrs during the process of *in vitro* fermentation. Undegraded residue was immediately filtered through 2 layers of nylon cloth (40-um pore size), and the gas was artificially collected with plastic syringe for CH_4 determination. The incubation solution of each sample was also sampled for ammonia nitrogen (NH_3-N) and volatile fatty acid (VFA) determination. Five milliliters of gas sample were collected and injected into the vacuum flask (Labco Exetainer, UK) for CH_4 determination.

Chemical analyses

The filtered residue was dried at 105°C for 12 h and weighed for dry matter disappearance determination. Neutral detergent fiber (NDF) content in the dried residue was determined according to the described method of Van Soest *et al.* (1991). Two milliliters of incubation solution was centrifuged at 10000×g and 4°C for 15 min, then 1.5 mL of supernatant solution was taken and 0.15 mL metaphosphoric acid was added and homogenized. The mixed solution was centrifuged at 10000×g and 4°C for 15 min

again, and the supernatant solution was used to determine VFA content with a gas chromatograph (HP5890, Agilent 5890; Agilent Technologies Co. Ltd, USA). A DB-FFAP column (30m in length with a 0.25mm i.d.) was used for the separation. The attenuation was set at a nitrogen diffluent ratio of 1:50, hydrogen flow was 30 mL/min, airflow was 365 mL/min, injector temperature was 250°C, column temperature was 150°C and detector temperature was 220°C. The N_2 was used as carrier gas at a flow rate of 0.8 mL/min. The relative response factor, representing the peak of each VFA, was calculated using the standard VFA mixture, which was chromatographed with each group of 10 samples. Total molar concentration was calculated by taking the sum of individual VFA as 100%.

For NH_3-N, 5 mL of incubation solution was centrifuged at 4000×g and 4°C for 10 min, then 2 mL of the supernatant solution was taken and mixed with 8 mL 0.2 M HCl into a tube followed by homogenization. Subsequently, 0.4 mL of the mixed solution was taken and mixed with 2 mL of sodium nitroprusside solutions (0.08 g sodium nitroprusside dissolved in 100 ml of 14% natrium salicylicum) and 2 mL of prepared solution (2 mL sodium hypochlorite solution mixed with 100 mL 0.3 M sodium hydroxide solution) into a tube followed by homogenization and equilibrated at room temperature for 10 min. The ultraviolet absorption value was recorded at 700 nm. The preparation of NH_4Cl standard solution was the same as above-mentioned procedures. The CH_4 analysis was performed by GC-flame ionization detection (FID) using gas chromatography (GC7890A, Agilent, America) equipped with a Hayesep Q packing column (2.44 M×1/8 in.×2.0 mm ID). The temperature of column and injector was respectively set at 60°C and 100°C, and held for 3 min. The N_2 was used as carrier gas at a flow rate of 21 mL/min.

Calculation and statistical analysis

During the initial stages of this work, the correlativity between the pressure in bottle and gas volume was measured at 39°C, and the regression equation was then established:

$y = (x-0.816)/0.805$ ($n=20$, $R^{2=}0.999$, $P<0.0001$),

Where, y represents gas volume (mL), x is the pressure in bottle (kPa), 0.816 and 0.805 are constant. The measured pressure was then converted to gas production (mL). *In vitro* gas production at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48 and 72 hrs were fitted to Logistic-Exponential (Wang *et al.,* 2011):

$GP = Vf\ (1 - \exp\ (d - t\times k)) / (1+\exp\ (b - k\times t))$

Where, GP represents gas production at t time, Vf means the maximum gas production (mL), k represents gas production fraction (/h), b and d represent the shape of the gas production curve. The following equation: $T0.5 = In\ (\exp\ (b)+2\exp\ (d))/k$ (Wang *et al.,* 2011) was used to calculate the time ($T0.5$, h) when half of the maximum gas production reached. $FRD0 = k/1+\exp\ (b))$ (Wang *et al.,* 2013) was used to calculate the initial fractional rate of degradation (/h).

Gas production, DM and NDF disappearances were corrected by subtracting the values obtained for the blanks. Statistical analyses were performed using the MIXED procedures (SAS Institute 2001). Data were analysed separately by forage substrate as a completely randomized design. The fixed effects in the model were cellulase, dose, and the cellulase×dose interaction. Linear and quadratic effects of DR were analysed using orthogonal polynomial contrasts. Cubic effects of DR were not examined for inexplicability in biology. The IML procedure of SAS (2001) was used to correct the contrast coefficients of orthogonal polynomial. Least squares means are reported throughout the text, and significance was declared at $P<0.05$.

RESULTS

In vitro gas production parameters

For grasses, *in vitro* gas production parameters generally were not affected by EC source, except for *Vf* which increased ($P<0.001$) by 8% and 19% for grasses supplemented with NP than those supplemented with TL and TR, respectively, and the grasses supplemented with NP had the maximum *Vf* value (Table 1). Only *Vf* and *k* were influenced by the dose rate of supplemented EC. The effects of TR (linear, $P<0.001$) and NP (quadratic, $P<0.05$) inclusion on *Vf*, and TL (linear, $P=0.01$) and NP (linear, $P=0.006$) inclusion on *k* were dose-dependent. The optimum DR for NP which was considered to be the minimum dose required to elicit the greatest significant increase in gas production and gas production rate compared with the control, being at 12 IU/g of DM.

For crop straws, the EC source affected *Vf* ($P<0.001$) and *d* ($P=0.02$) values, and the lowest *Vf* was observed in TR. Relative improvements in *Vf* were 17% and 9% for TL and NP compared with TR. The effect of all EC addition on *in vitro* gas production parameters, except for *RFD0*, depended upon the DR used, as proved by significant EC×DR interactions (Table 2). The linear effects on *Vf*, *k*, *b* and *d* values to DR were found for TL, and a linear effects on *Vf*, *d* and *T0.5* values to DR were also found for NP. A linear response on *Vf*, a quadratic response on *k* and *d* values to DR were observed for TR, respectively. The optimum DR varied among EC. Low DR of NP (12 IU/g of DM) and the highest DR of TL (62 IU/g of DM) increased *Vf* by 5% and 7.0% compared with control.

DM and NDF disappearance

For grasses, the effect of each of 3 EC on DMD depended on the source of EC and the DR used, while for NDFD, EC supplementing effects were only related to DR used (Table 3). The source of EC affected ($P<0.001$) DMD, and the lowest DMD was noted in TL; NDFD in TL also decreased by margin of 7.1% and 5.5% compared with TR and NP, respectively. A quadratic response on DMD and NDFD to DR was observed for all EC except for NDFD of NP which linearly ($P=0.01$) increased NDFD of grasses. Degradation of DM and NDF of grasses were increased when EC was

supplemented. Optimum DR for improving DMD and NDFD was 12 IU/g of DM for TL, TR and NP compared with control. Improvements in DMD were 26.7, 28.1 and 24.6%, and in NDFD were 17, 24.4 and 24%, respectively.

Table 1. *In vitro* fermentation characteristics of grass supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Vf, mL	TL	274[f]	281	266	272	284	8.4	P<0.001	NS	P<0.001
	TR	247[g]	281[a]	260[ab]	245[ab]	236[b]			L (P<0.001)	
	NP	295[e]	281	326	272	287			NS	
	SEM[ŧ]	5.3								
k,/h	TL	0.065	0.059[b]	0.058[ab]	0.068[a]	0.068[a]	0.0051	NS	L (P=0.01)	NS
	TR	0.062	0.059	0.050	0.067	0.068			NS	
	NP	0.069	0.059[ab]	0.061[ab]	0.064[ab]	0.082[a]			L (P=0.006)	
	SEM[ŧ]	0.00274								
b	TL	-1.78	-2.03	-2.31	-1.18	-1.86	1.333	NS	NS	NS
	TR	-1.33	-2.03	-3.73	-0.01	-0.25			NS	
	NP	-0.98	-2.03	-0.45	-3.11	0.63			NS	
	SEM[ŧ]	0.6587								
d	TL	-0.126	-0.183	-0.182	-0.087	-0.109	0.0487	NS	NS	NS
	TR	-0.106	-0.183	-0.056	-0.137	-0.126			NS	
	NP	-0.123	-0.183	0.001	-0.150	-0.221			NS	
	SEM[ŧ]	0.0191								
T0.5, h	TL	15.3	16.3	15.7	15.3	14.7	1.13	NS	NS	NS
	TR	14.9	16.3	15.3	14.6	14.8			NS	
	NP	15.5	16.3	16.8	13.5	16.1			NS	
	SEM[ŧ]	0.6485								
FRD0,/h	TL	0.036	0.029	0.034	0.036	0.037	0.0052	NS	NS	NS
	TR	0.037	0.029	0.040	0.034	0.036			NS	
	NP	0.034	0.029	0.034	0.040	0.027			NS	
	SEM[ŧ]	0.0029								

[e-g]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-b]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]*Vf*=the maximum gas production; *k*=gas production fraction; *b* and *d*=the shape of the gas production curve; *T0.5*=the time when half of the maximum gas production reached; *FRD0*=the initial fractional rate of degradation;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

Table 2. *In vitro* fermentation characteristics of straws supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Vf, mL	TL	275[c]	274[ab]	243[b]	290[ab]	293[a]	10.8	P<0.001	L (P=0.03)	P<0.001
	TR	235[f]	274[a]	263[ab]	216[b]	227[ab]			L (P<0.001)	
	NP	259[e]	274[ab]	289[a]	238[b]	251[ab]			L (P=0.01)	
	SEM[ŧ]	6.1								
k,/h	TL	0.059	0.062[a]	0.061[ab]	0.054[ab]	0.052[b]	0.0036	NS	L (P=0.04)	P<0.001
	TR	0.061	0.062[b]	0.043[c]	0.083[a]	0.057[b]			Q (P<0.001)	
	NP	0.061	0.062	0.057	0.061	0.065			NS	
	SEM[ŧ]	0.00225								
b	TL	0.402	0.798[a]	0.734[a]	0.383[ab]	0.091[b]	0.2509	NS	L (P=0.01)	P<0.001
	TR	0.392	0.798	-0.807	1.398	0.586			NS	
	NP	0.580	0.798	0.686	0.537	0.518			NS	
	SEM[ŧ]	0.1631								
d	TL	-0.214[e]	-0.147[a]	-0.418[b]	-0.114[a]	-0.111[a]	0.0528	P=0.02	L (P<0.001)	P<0.001
	TR	-0.358[f]	-0.147[a]	-0.153[a]	-0.674[b]	-0.248[a]			Q (P<0.001)	
	NP	-0.259[ef]	-0.147[a]	-0.142[a]	-0.242[ab]	-0.393[b]			L (P=0.01)	
	SEM[ŧ]	0.034								
T0.5, h	TL	21.4	22.0	20.6	21.8	21.7	0.73	NS	NS	P=0.01
	TR	20.5	22.0	20.2	20.3	21.2			Q (P=0.04)	
	NP	20.7	22.0[a]	23.3[a]	19.7[b]	19.2[b]			L (P<0.001)	
	SEM[ŧ]	0.4535								
FRD0,/h	TL	0.021	0.019	0.019	0.022	0.022	0.0015	NS	NS	P=0.01
	TR	0.020	0.019	0.024	0.016	0.020			NS	
	NP	0.021	0.019	0.019	0.022	0.022			NS	
	SEM[ŧ]	0.0009								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]*Vf*=the maximum gas production; *k*=gas production fraction; *b* and *d*=the shape of the gas production curve; *T0.5*=the time when half of the maximum gas production reached; *FRD0*=the initial fractional rate of degradation;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

For crop straws, EC source only affected (P<0.001) DMD (Table 3). Difference in NDFD among 3 EC was not observed. Crop straws supplemented with EC from TR and NP increased DMD by 16.9% and 14.5% compared with those supplemented with EC from TL. All of 3 EC influence on DMD and NDFD relied on the DR used, as evidenced by significant EC×DR interaction (Table 3). A quadratic response on DMD and NDFD to DR was observed for all EC. The optimum DR in increasing DMD was 12 IU/g of DM for 3 EC, and in improving NDFD was 12 IU/g of DM for TL and NP, and 37 IU/g of DM for TR. The lowest DR of TL, TR and NP (12 IU/g of DM) increased DMD by 21.2, 19.2 and 15% compared with control. Improvements in the NDFD were 23.8% for TL, 25.7% for NP, and 31.7% for TR.

Table 3. *In vitro* disappearance of DM and NDF of forage supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Grass										
DMD, %	TL	47.2[f]	35.1[c]	61.8[a]	39.3[bc]	40.6[b]	1.82	P<0.001	Q (P=0.01)	P<0.001
	TR	61.0[e]	35.1[b]	63.2[a]	60.6[a]	59.1[a]			Q (P<0.001)	
	NP	60.6[e]	35.1[b]	59.7[a]	59.1[a]	63.0[a]			Q (P<0.001)	
	SEM[t]	1.20								
NDFD, %	TL	76.8	57.6[b]	74.6[a]	76.1[a]	79.7[a]	4.18	NS	Q (P=0.03)	NS
	TR	83.9	57.6[b]	82.0[a]	87.6[a]	82.3[a]			Q (P<0.001)	
	NP	82.3	57.6[c]	81.6[ab]	75.5[b]	89.7[a]			L (P=0.01)	
	SEM[t]	2.46								
Straw										
DMD, %	TL	35.4[f]	32.5[b]	53.7[a]	26.0[c]	26.5[c]	1.87	P<0.001	Q (P=0.02)	P<0.001
	TR	52.3[e]	32.5[b]	51.7[a]	53.5[a]	51.8[a]			Q (P<0.001)	
	NP	49.9[e]	32.5[b]	47.5[a]	51.2[a]	50.9[a]			Q (P<0.001)	
	SEM[t]	1.20								
NDFD, %	TL	60.3	39.4[b]	63.2[a]	61.9[a]	55.7[a]	3.30	NS	Q (P<0.001)	P=0.02
	TR	59.4	39.4[c]	55.2[b]	65.1[a]	58.0[ab]			Q (P<0.001)	
	NP	66.2	39.4[c]	71.1[a]	58.6[b]	69.0[a]			Q (P=0.02)	
	SEM[t]	1.91								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]DMD=DM degradability; NDFD=NDF degradability;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[t]SEM for pooled mean of EC excluding the dose rate of 0.

pH, NH_3-N and CH_4 production

The source of EC influenced pH value (P<0.001), concentration of NH_3-N in fermentation liquor (P=0.001) and CH_4 production (P<0.001) of grasses (Table 4). Grasses supplemented with EC of TR and NP had lower pH value, NH_3-N concentration and CH_4 production than those supplemented with EC of TL. The decreases in pH were 3.8% and 3.2%, in NH_3-N were 28% and 39%, and in CH_4 were 44% and 24% for TR and NP compared with TL. Dosage of each 3 of EC influenced pH value, NH_3-N and CH_4 production of grasses. The pH of TR and NP decreased, while that of TL increased. The optimum DR of 3 EC for decreasing pH, NH_3-N and CH_4 was 12 IU/g of DM for TL, TR and NP. Relative decreases in pH, NH_3-N and CH_4 were 6.2%, 65% and 48% for TL, 6.3%, 52% and 51% for TR, and 4.2%, 71% and 31% for NP, respectively.

Table 4. Ammonia nitrogen concentration and pH value of fermentation liquor of forage supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Grass										
pH	TL	6.80[e]	6.93[a]	6.50[b]	6.94[a]	6.96[a]	0.023	P<0.001	Q (P<0.001)	P<0.001
	TR	6.54[f]	6.93[a]	6.49[b]	6.58[b]	6.55[b]			Q (P<0.001)	
	NP	6.58[f]	6.93[a]	6.64[b]	6.55[b]	6.56[b]			Q (P<0.001)	
	SEM[t]	0.013								
NH_3-N,	TL	155[e]	195[a]	69[b]	196[a]	199[a]	16.9	P=0.01	L (P=0.03)	P=0.01
mg/L	TR	111[f]	195[a]	93[b]	102[b]	138[b]			Q (P<0.001)	
	NP	95[f]	195[a]	57[c]	115[b]	114[b]			Q (P=0.01)	
	SEM[t]	10.4								
CH_4,	TL	5.20[e]	6.19[a]	3.23[b]	6.28[a]	6.08[a]	0.428	P<0.001	Q (P<0.001)	P<0.001
mmol/g DMD	TR	2.91[g]	6.19[a]	3.03[b]	2.90[b]	2.81[b]			Q (P<0.001)	
	NP	3.94[f]	6.19[a]	4.28[b]	3.83[b]	3.71[b]			Q (P=0.02)	
	SEM[t]	0.219								
Straw										
pH	TL	6.77[e]	6.87[a]	6.44[b]	6.94[a]	6.93[a]	0.019	P<0.001	Q (P<0.001)	P<0.001
	TR	6.53[g]	6.87[a]	6.48[b]	6.54[b]	6.57[b]			Q (P<0.001)	
	NP	6.60[f]	6.87[a]	6.73[b]	6.52[c]	6.54[c]			Q (P<0.001)	
	SEM[t]	0.011								
NH_3-N,	TL	143[e]	227[a]	74[c]	202[a]	153[b]	16.6	P=0.005	Q (P<0.001)	P<0.001
mg/L	TR	99[f]	227[a]	109[b]	89[b]	98[b]			Q (P<0.001)	
	NP	97[f]	227[a]	76[b]	96[b]	119[b]			Q (P<0.001)	
	SEM[t]	6.4								
CH_4,	TL	7.20[e]	7.75[a]	3.31[b]	9.15[a]	9.13[a]	0.518	P<0.001	L (P<0.001)	P<0.001
mmol/g DMD	TR	3.07[g]	7.75[a]	3.47[b]	2.79[b]	2.97[b]			Q (P<0.001)	
	NP	3.90[f]	7.75[a]	4.45[b]	3.71[b]	3.55[b]			Q (P<0.001)	
	SEM[t]	0.239								

[c-g]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]NH_3-N=ammonia nitrogen concentration; CH_4=methane production;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum* (for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum* (for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC; [¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[t]SEM for pooled mean of EC excluding the dose rate of 0.

For crop straws, the differences of EC source on pH value (P<0.001), NH_3-N (P=0.005) and CH_4 (P<0.001) were observed among 3 EC (Table 4). The favorable EC in reducing pH, NH_3-N and CH4 was TR and NP which decreased pH by 3.7% and 2.6%, reduced NH_3-N by 44% and 47%, and lower CH_4 by 134% and 85% compared with TL, respectively. Dose rate response in pH, NH_3-N and CH_4 varied among EC, as evidenced by significant EC×DR interaction. Optimum DR for decreasing pH, NH_3-N and CH_4 was 12 IU/g of DM for TL, TR and NP, except for pH of NP which was 37

IU/g of DM was favorable. Relative decreases in NH_3-N and CH_4 at this DR were 67, 52 and 67% and were 57, 55 and 43%, and in pH at those DR were 6.3, 5.7 and 5.1% for TL, TR and NP compared with control, respectively.

Volatile fatty acids

For grasses, the source of EC affected volatile fatty acid concentration in fermentation liquor and the ratio of acetate to propionate, except for isobutyrate (Table 5). The grasses supplemented with EC of NP had the highest TVFA, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate and the ratio of acetate to propionate. The EC of NP improved TVFA by 29 and 22%, enhanced acetate by 51 and 31%, and increased the ratio of acetate to propionate by 16% and 17% compared with TL and TR, and enhanced butyrate, isovalerate and valerate by 36, 22 and 25% compared with TR, and increased propionate by 26% compared with TL, respectively. The EC produced variable effects on short chain fatty acid across DR, indicated by the EC×DR interaction. Optimum DR for increasing TVFA and each volatile fatty acid was 37 IU/g of DM for NP compared with control, and relative improvements in these values at this DR were ranged from 20% of isobutyrate to 106% of acetate. Optimum DR for increasing acetate and propionate was 37 IU/g of DM for TR compared with control. Relative improvements in these values at this DR were 41% and 35%. The EC of TL supplementation did not increase the concentration of TVFA, each volatile fatty acid compared to control.

For crop straws, the source of EC had significant effects on TVFA ($P<0.001$), acetate ($P<0.001$), butyrate ($P<0.001$), isovalerate ($P=0.01$) and the value of A:P ($P<0.001$). The highest TVFA, acetate, butyrate and the ratio of acetate to propionate were observed in NP, and the highest isovalerate was noted in the EC of TR. The EC of NP increased ($P<0.001$) TVFA by 21% and 23%, enhanced acetate by 47% and 37% compared with TL and TR, respectively, and increased propionate by 14% compared with TL. More than 28% and 34% of butyrate, and 20% and 19% of isovalerate were produced when crop straws supplemented with the EC of TR and NP compared with those of TL. The EC produced variable effects on short chain fatty acid across DR, indicated by the EC×DR interaction. whereas the improvements in these parameters were very weak for crop straws supplemented with EC of TL and TR compared with control. The EC of NP linearly increased ($P<0.001$) TVFA, butyrate, isovalerate, valerate and the ratio of acetate to propionate, and quadratically increased acetate ($P=0.02$) and propionate ($P=0.04$) compared with control. Optimum DR for increasing the value of these parameters was 37 IU/g of DMD, except for A:P, and relative improving in the values of these parameters at this DR were ranged from 21% of valerate to 75% of acetate compared with control. The EC supplementation could not decrease the ratio of acetate to propionate.

Table 5. Volatile fatty acid concentration (mmol/l) in incubation solution of grasses supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[Ŧ]		
		Mean	0	12	37	62		EC	DR	EC×DR
TVFA	TL	18.1^{f}	17.4	18.2	16.3	19.8	1.49	P<0.001	NS	P<0.001
	TR	19.1^{f}	17.4^{ab}	19.8^{ab}	20.5^{a}	17.1^{b}			Q (P=0.02)	
	NP	23.3^{e}	17.4^{c}	18.7^{bc}	28.1^{a}	23.2^{ab}			Q (P=0.02)	
	SEM[ŧ]	0.95								
Acetate	TL	6.35^{f}	5.81	6.49	5.69	6.86	0.776	P<0.001	NS	P=0.004
	TR	7.30^{f}	5.81^{b}	7.36^{ab}	8.21^{a}	6.35^{b}			Q (P=0.003)	
	NP	9.57^{e}	5.81^{c}	7.90^{b}	11.95^{a}	8.86^{b}			Q (P=0.003)	
	SEM[ŧ]	0.497								
Propionate	TL	5.74^{f}	5.56	6.17	4.93	6.13	0.453	P=0.007	NS	P=0.01
	TR	6.66^{ef}	5.56^{b}	6.34^{b}	7.51^{a}	6.14^{b}			Q (P=0.002)	
	NP	7.22^{e}	5.56^{b}	6.48^{ab}	7.71^{a}	7.46^{a}			L (P=0.02)	
	SEM[ŧ]	0.296								
Butyrate	TL	3.46^{c}	3.38^{ab}	3.18^{b}	3.27^{b}	3.92^{a}	0.219	P<0.001	L (P=0.03)	P<0.001
	TR	2.79^{f}	3.38^{a}	3.62^{a}	2.49^{b}	2.25^{b}			L (P<0.001)	
	NP	3.80^{e}	3.38^{b}	2.45^{c}	4.92^{a}	4.02^{b}			L (P<0.001)	
	SEM[ŧ]	0.138								
Isobutyrate	TL	0.788	0.825^{ab}	0.684^{b}	0.750^{b}	0.929^{a}	0.0451	NS	Q (P=0.004)	P<0.001
	TR	0.775	0.825	0.665	0.819	0.840			NS	
	NP	0.759	0.825^{b}	0.460^{c}	0.990^{a}	0.827^{b}			L (P=0.003)	
	SEM[ŧ]	0.0273								
Isovalerate	TL	0.933^{ef}	0.975^{ab}	0.839^{b}	0.880^{b}	1.079^{a}	0.0673	P=0.02	Q (P=0.009)	P<0.001
	TR	0.803^{f}	0.975^{a}	0.976^{a}	0.680^{b}	0.754^{b}			L (P=0.002)	
	NP	0.973^{e}	0.975^{b}	0.606^{c}	1.311^{a}	1.003^{b}			L (P=0.01)	
	SEM[ŧ]	0.0409								
Valerate	TL	0.839^{ef}	0.836	0.820	0.771	0.925	0.0717	P=0.01	NS	P=0.005
	TR	0.791^{f}	0.836	0.872	0.744	0.758			NS	
	NP	0.987^{e}	0.836^{b}	0.804^{b}	1.170^{a}	0.987^{ab}			L (P=0.04)	
	SEM[ŧ]	0.0452								
A:P	TL	1.08^{f}	1.04^{ab}	1.00^{b}	1.14^{a}	1.12^{a}	0.062	P=0.003	L (P=0.03)	P<0.001
	TR	1.07^{f}	1.04	1.10	1.10	1.01			NS	
	NP	1.25^{e}	1.04^{b}	1.19^{ab}	1.43^{a}	1.14^{b}			Q (P=0.003)	
	SEM[ŧ]	0.036								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]TVFA=Total volatile fatty acid; A:P=ration of acetic acid to propionic acid;

[‡]TL, TR and NP were cellulose originating from *Trichoderma longibrachiatum* (for TL), *T. reesei* (for TR) or *Neocallimastix patriciarum* (for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[Ŧ]EC=exogenous cellulase; DR=dose rate; L=linear effect of DR; Q=quadratic effect of DR; NS=no significant; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

Table 6. Volatile fatty acid concentration (mmol/l) in incubation solution of straws supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[Ŧ]		
		Mean	0	12	37	62		EC	DR	EC×DR
TVFA	TL	14.9[f]	14.7	17.8	13.3	13.8	1.01	P<0.001	NS	P<0.001
	TR	15.1[f]	14.7	16.2	14.3	14.8			NS	
	NP	18.3[e]	14.7[b]	14.3[b]	21.3[a]	19.4[a]			L (P<0.001)	
	SEM[ŧ]	0.61								
Acetate	TL	5.53[f]	4.98	6.49	4.90	5.20	0.471	P<0.001	NS	P<0.001
	TR	5.16[f]	4.98	5.66	4.88	4.95			NS	
	NP	7.60[e]	4.98[b]	5.84[b]	8.71[a]	8.25[a]			Q (P=0.02)	
	SEM[ŧ]	0.288								
Propionate	TL	5.21	4.91	5.95	4.61	5.05	0.333	NS	NS	P=0.02
	TR	4.88	4.91[ab]	5.65[a]	4.37[b]	4.62[b]			L (P=0.02)	
	NP	5.57	4.91[b]	5.25[ab]	6.17[a]	5.28[ab]			Q (P=0.04)	
	SEM[ŧ]	0.204								
Butyrate	TL	2.33[f]	2.81[a]	3.26[a]	1.91[b]	1.81[b]	0.169	P<0.001	L (P<0.001)	P<0.001
	TR	2.98[e]	2.81	3.03	2.93	2.97			NS	
	NP	3.13[e]	2.81[b]	2.02[c]	3.90[a]	3.46[ab]			L (P<0.001)	
	SEM[ŧ]	0.093								
Isobutyrate	TL	0.647	0.677	0.595	0.687	0.660	0.0337	NS	NS	P<0.001
	TR	0.649	0.677[a]	0.549[b]	0.676[a]	0.722[a]			Q (P=0.03)	
	NP	0.646	0.677[a]	0.363[b]	0.780[a]	0.795[a]			L (P<0.001)	
	SEM[ŧ]	0.0185								
Isovalerate	TL	0.645[f]	0.714[ab]	0.822[a]	0.594[bc]	0.518[c]	0.0425	P=0.01	NS	P<0.001
	TR	0.771[e]	0.714	0.730	0.772	0.810			NS	
	NP	0.766[e]	0.714[b]	0.424[c]	0.976[a]	0.897[a]			L (P<0.001)	
	SEM [6]	0.0243								
Valerate	TL	0.573	0.636	0.637	0.559	0.523	0.0362	NS	NS	P<0.001
	TR	0.642	0.636[ab]	0.562[b]	0.670[a]	0.693[a]			L (P=0.008)	
	NP	0.621	0.636[b]	0.399[c]	0.770[a]	0.694[ab]			L (P<0.001)	
	SEM[ŧ]	0.0204								
A:P	TL	1.06[f]	0.995	1.06	1.09	1.02	0.046	P<0.001	NS	P<0.001
	TR	1.05[f]	0.995[ab]	0.984[b]	1.11[a]	1.06[ab]			L (P=0.02)	
	NP	1.29[e]	0.995[b]	1.05[b]	1.38[a]	1.45[a]			L (P<0.001)	
	SEM[ŧ]	0.025								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]SCFA=short chain fatty acid; A:P=ration of acetic acid to propionate;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *T. reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[Ŧ]EC=exogenous cellulase; DR=dose rate; L=linear effect of DR; Q=quadratic effect of DR; NS=no significant; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

DISCUSSION

Cell wall degrading enzymes used in ruminants feedstuffs may differ in improving the degradation of forage because of the composition and the activity of enzyme. The maximum gas production of grass and crop straw differed among three EC was similar to that of Eun and Beauchemin (2007b) and Chen *et al.* (2013). Eun and Beauchemin (2007b) reported that feed enzymes originated from *Trichoderma longibrachiatum* had higher gas production than that from *Penicillum funiculosum*. In present study, crop straw or grass supplemented with EC of TL and NP had higher gas production than that of TR. Also, Chen *et al.* (2013) found that gas production at 48 h incubation time ranged from 166 to 177 mL/g for corn silage added 5 cellulase. Compose of exoglucanase, endoglucanase and xylanase, and their activity in enzyme system may be responsible for this difference. It indicated that the origination of EC should be considered when EC was used to improve *in vitro* fermentation of forage. When the gas production were fitted to the Wang *et al.* (2011) model, no obvious improvement in the maximum gas production compared with control was consistent with that of previous studies (Colombatto *et al.,* 2004; Eun and Beauchemin, 2007b; Chen *et al.,* 2013). Index of *FRD0* and *T0.5* reflect the rate of fermentation at early incubation stages of '< 12 h' and the incubation time of half of the maximum gas production reached, respectively. Supplementation of EC, although, did not increase the value of *T0.5* and *FRD0* obviously, a numerically decrease in *T0.5* and an increase in *FRD0* indicated that EC supplementation may be beneficial to improve the effect of bacteria on degradation of forage. In the other hand, *T0.5* is more than 14 h for grass and 20 h for crop straw indicated mainly degradation period of bacteria on forage is in the middle or late stages of incubation. These results also support the general agreement that enzymes increased the rate, but not the extent, of feed degradation in the rumen (Beauchemin *et al.,* 2001).

Analyzing on the data of DMD and NDFD revealed that enzyme treatments increased the degradable fraction of the forages, which is agreement with the data obtained from grass (Zhu *et al.,* 1999), alfalfa (Chen *et al.,* 1994; Eun and Beauchemin, 2007b), corn silage (Eun and Beauchemin, 2007b; Sun *et al.,* 2009; Chen *et al.,* 2013), and with our previous report (Tang *et al.,* 2008). Other studies using maize silage or corn silage have reported small increases (Chen *et al.,* 1994; Eun and Beauchemin, 2007b). Our results suggest that more macromolecular were hydrolyzed to simpler and degradable ones as enzymes were supplemented in forages. Origin of EC differed in improving DMD and NDF was also observed in previous studies (Colombatto *et al.,* 2004; Eun and Beauchemin, 2007b). Probable cause may be related to the activity of endoglucanase which hydrolyze cellulose chains at random, and exoglucanase which hydrolyze cellulose chain from the nonreducing end (Bhat and Hazlewood, 2001), in the enzyme system. Eun and Beauchemin (Eun and Beauchemin 2007b) also found endoglucanase linked to NDF degradability of alfalfa and corn silage. Significant increase in DMD or NDFD indicated catalytic affect of EC on the substrate

(Morgavi *et al.,* 2001). In any case, the outcome indicated that the fermentation efficiency of forage may be improved as enzyme used as additives in ruminant feedstuffs.

The value of pH is a main factor in reflecting internal environment of rumen. Treatments of TR and NP or EC reduced pH value of fermentation liquor to 6.49 and 6.73 compared with TL, or control suggests that TR and NP, or EC treatments could maintain more suitable condition for fermentation, and suitable for the growth of micro-organism. Final pH value of EC treatment ranged from 6.53 to 6.80, and can be considered optimal for fiber degradation in the rumen (Stewart *et al.,* 1997). TR and NP or EC treatments in forage had higher DMD than TL or control may partially due to this reason. Satter and Slyter (1974) suggested that the lowest ammonia nitrogen concentration of rumen liquor should be higher than 5 mL/dL for bacteria to get the highest growth rate. Concentration of NH_3-N in EC treatments is higher than 5 mg/dL indicated the growth rate of bacteria will not be restricted. In the other hand, ammonia nitrogen is a main source of nitrogen in the synthesis of rumen bacteria, and 18% to 100% of bacterial nitrogen is originated from ammonia nitrogen (Salter *et al.,* 1979). The treatments of EC or TR and NP had lower NH_3-N concentration than control or TL implied that forage supplemented EC or EC of TR and NP could enhance the utilization of bacteria on nitrogen.

In present study, EC or EC of TR and NP treatment significantly decreased methane production compared with control or TL. Giraldo *et al.* (2007) found that methane production was not influenced for cellulase was sprayed into the diet which was composed with forage and concentration at 70: 30. Whereas, Chung *et al.* (2012) reported that methane production, whether calculated as per kg of DM or per kg of milk, would increase when enzyme added in dairy diets at 0.5 and 1.0 mL/kg. Dong *et al.* (1999) also found methane production increased by 43% when cellulase and xylanase were added in hay. Beauchemin *et al.* (2008; 2009) reported that enzyme supplementation though absolutely increased methane production, methane production per kg of milk would decrease. Methane production decrease may be related to microflora change of methanogenium leaded by enzyme addition (Zhou *et al.,* 2011). Addition of EC leaded to methane production decrease suggested that utilization of cellulase, especially for TR and NP, in ruminant diets may be an efficient method in reducing greenhouse effect caused by CH_4 emitted from ruminant production.

Changes in TVFA and each profile of VFA corresponded to increased fiber degradation of forage. Addition of EC increased the concentration of TVFA of forage was consistent with that of previous studies (Eun and Beauchemin, 2007a; Eun and Beauchemin, 2007b; Eun and Beauchemin, 2008; Giraldo *et al.,* 2008). This indicated that the activity of bacteria in degrading fiber has been promoted by EC, especially for EC of NP. The ratio of acetate to propionate of EC treatment was not decreased, or even increased compared with control was inconsistent with that of previous studies

(Eun and Beauchemin, 2007a; 2008; Giraldo *et al.,* 2008). Eun and Beauchemin (2007b) found that the addition of single or combined enzyme numerically decreased the ratio of acetate to propionate of alfalfa. The inconsistent of enzyme addition on the changes of VFA composition may relate to the enzyme activities added, the forage substrates, diet of donor animal and donor species used. The ratio of acetate to propionate was lower for forage supplemented with EC at 37 IU/g of DM than that of higher dosage indicated that it is benefit for diets supplementing EC at lower dosage from increasing availability of glucogenic precursors to ruminants.

CONCLUSIONS

Exogenous cellulase originated from TR and NP improved *in vitro* DMD of forage, decreased pH value, NH_3-N and CH_4 production of forage compared to TL. Forage added with EC of NP had higher TVFA, acetate, propionate and the ratio of acetate to propionate compare with TL and TR. Forage supplemented with EC could improve DMD, NDFD and TVFA, and decrease pH value, NH_3-N and CH_4 production, and their optimum DR varied hardly depending upon the forage. In general, low DR of EC resulted in increase in DMD and NDFD and decrease in CH_4 and NH_3-N as that of medium and higher DR. It is recommended that the treatments of TR and NP should be further evaluated in animal feeding study.

ACKNOWLEDGEMENTS

We are grateful to International Atomic Energy Agency (16315), Chinese Academy of Sciences (XDA05020700) and National Science and Technology Support Program (2012BAD12B02-3) for providing the financial support for this study.

REFERENCES

Beauchemin, K.A., Kreuzer, M., OMara, F. and McAllister, T.A. 2008. Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture,* 48: 21-27.

Beauchemin, K.A., McAllister, T.A. and McGinn, S.M. 2009. Dietary mitigation of enteric methane from cattle. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources,* 4: 1-18.

Beauchemin, K.A., Morgavi, D.P., McAllister, T.A., Yang, W.Z. and Rode, L.M. 2001. The use of feed enzymes in ruminant diets, In: *Recent Advances in Animal Nutrition* (Eds. P.C. Garnsworthy and P.J. Wiseman), Nottingham University, Nottingham, UK, 297-322.

Bhat, M.K. and Hazlewood, G.P. 2001. Enzymology and other characteristics of cellulases and xylanases, In: *Enzymes in Farm Animal Nutrition* (Eds. M.R. Bedford and G.G. Partridge), CABI Publishing, Wallingford, UK, 11-60.

Burroughs, W., Woods, W., Ewing, S.A., Greig, J. and Theurer, B. 1960. Enzyme additions to fattening cattle rations. *Journal of Animal Science,* 19: 458-464.

Chen, J., Stokes, M.R. and Wallace, C.R. 1994. Effects of enzyme-inoculant systems on preservation and nutritive value of haycrop and corn silages. *Journal of Dairy Science,* 77: 501-512.

Chen, X., Mao, H.L., Wang, J.K., Wu, C.H. and Liu, J.X. 2013. Exogenous fibrolytic enzymes: effects on *in vitro* fermentation and methane production of corn silage. *Chinese Journal of Animal Nutrition,* 25: 214-221.

Chung, Y.H., Zhou, M., Holtshausen, L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M. and Beauchemin, K.A. 2012. A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions. *Journal of Dairy Science,* 95: 1419-1427.

Colombatto, D., Mould, F.L., Bhat, M.K., Phipp, R.H. and Owen, E. 2004. *In vitro* evaluation of fibrolytic enzymes as additives for maize (*Zea mays* L.) silage III. Comparison of enzyme derived from psychrophilic, mesophilic or thermophilic sources. *Animal Feed Science and Technology,* 111: 145-159.

Ding, X., Long, R., Zhang, Q., Huang, X., Guo, X. and Mi, J. 2012. Reducing methane emissions and the methanogen population in the rumen of Tibetan sheep by dietary supplementation with coconut oil. *Tropical Animal Health and Production,* 44: 1541-1545.

Dong, Y., Bae, H.D., McAllister, T.A., Mathison, G.W. and Cheng, K.J. 1999. Effects of exogenous fibrolytic enzymes, α-bromoethanesulfonate and monensin on fermentation in a rumen simulation (Rusitec) system. *Canada Journal of Animal Science,* 79: 491-498.

Eun, J.S. and Beauchemin, K.A. 2007a. Assessment of the efficacy of varying experimental exogenous fibrolgytic enzymes using *in vitro* fermentation characteristics. *Animal Feed Science and Technology,* 132: 298-315.

Eun, J.S. and Beauchemin, K.A. 2007b. Enhancing In Vitro Degradation of Alfalfa Hay and Corn Silage Using Feed Enzymes. *Journal of Dairy Science,* 90: 2839-2851.

Eun, J.S. and Beauchemin, K.A. 2008. Assessment of the potential of feed enzyme additives to enhance utilization of corn silage fibre by ruminants. *Canadian Journal of Animal Science,* 88: 97-106.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2007. Effects of exogenous cellulase supplementation on microbial growth and ruminal fermentation of a high-forage diet in Rusitec fermenters. *Journal of Animal Science,* 85: 1962-1970.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2008. Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage: concentrate ratios. *Animal Feed Science and Technology,* 141: 306-325.

Malik, R. and Bandla, S. 2010. Effect of source and dose of probiotics and exogenous fibrolytic enzymes (EFE) on intake, feed efficiency, and growth of male buffalo (*Bubalus bubalis*) calves. *Tropical Animal Health and Production,* 42: 1263-1269.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., McAllister, T.A., Iwassa, A.D., Wang, Y. and Yang, W.Z. 2001. Resistence of feed enzymes to proteolytic inactivation by rumen micro-organisms and gastrointestinal proteases. *Journal of Animal Science,* 79: 1621-1630.

Nsereko, V.L., Beauchemin, K.A., Morgavi, D.P., Rode, L.M., Furtado, A.F., McAllister, T.A., Iwaasa, A.D., Yang, W.Z. and Wang, Y. 2002. Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. *Canadian Journal of Microbiology,* 48: 14-20.

Reddish, M.A. and Kung, L.Jr. 2007. The effect of feeding a dry enzyme mixture with fibrolytic activity on the performance of lactating cows and digestibility of a diet for sheep. *Journal of Dairy Science,* 90: 4724-4729.

Salter, D.N., Daneshvar, K. and Smith, R.H. 1979. The origin of nitrogen incorporated into compounds in the rumen bacteria of steers given protein- and urea- containing diets. *British Journal of Nutrition,* 41: 197-209.

SAS Institute. 2001. *The SAS System for Microsoft Windows, release 8.2*. SAS Institute Inc., Cary, NC.

Satter, L.D. and Slyter, L.L. 1974. Effect of ammonia concentration on rumen microbial protein production *in vitro*. *British Journal of Nutrition,* 32: 199-208.

Shojaeian, K. and Thakur, S.S. 2007. Effect of exogenous fibrolytic enzymes supplementation to substrates containing different roughage: concentrate ratios on *in vitro* rumen fermentation, DM and NDF degradability. *Indian Journal of Dairy Science,* 60: 94-101.

Stewart, C.S., Flint, H.J. and Bryant, M.P. 1997. The rumen bacteria, In: *The Rumen Microbial Ecosystem* (Eds. P.N. Hobson and C.S. Stewart), Chapman and Hall, London, UK, 10-72.

Sun, Z.H., Liu, S.M., Tayo, G.O., Tang, S.X., Tan, Z.L., Lin, B., He, Z.X., Hang, X.F., Zhou, Z.S. and Wang, M. 2009. Effects of cellulase or lactic acid bacteria on silage fermentation and *in vitro* gas production of several morphological fractions of maize stover. *Animal Feed Science and Technology,* 152: 219-231.

Sutton, J.D., Phipps, R.H., Beever, D.E., Humphries, D.J., Hartnell, G.F., Vicini, J.L. and Hard, D.L. 2003. Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in Holstein-Friesian cows. *Journal of Dairy Science,* 86: 546-556.

Tang, S., Tan, Z., Zhou, C., Jiang, H., Jiang, Y. and Sheng, L. 2006. A comparison of *in vitro* fermentation characteristics of different botanical fractions of mature maize stover. *Journal of Animal and Feed Sciences,* 15: 505-515.

Tang, S.X., Tayo, G.O., Tan, Z.L., Sun, Z.H., Shen, L.X., Zhou, C.S., Xiao, W.J., Ren, G.P., Han, X.F. and Shen, S.B. 2008. Effects of yeast culture and fibrolytic enzymes supplementation on *in vitro* fermentation characteristics of low-quality cereal straws. *Journal of Animal Science,* 86: 1164-1172.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3597.

Wang, M., Sun, X.Z., Tang, S.X., Tan, Z.L. and Pecheco, D. 2013. Deriving fractional rate of degradation of logistic-exponential (LE) model to evaluate early *in vitro* fermentation. *Animal,* 7: 920-929.

Wang, M., Tang, S.X. and Tan, Z.L. 2011. Modeling *in vitro* gas production kinetics: Derivation of Logistic-Exponential (LE) equations and comparison of models. *Animal Feed Science and Technology,* 165: 137-150.

Zhou, M., Chung, Y.H., Beauchemin, K.A., Holtshausen, L., Oba, M., McAllister, T.A. and Guan, L.L. 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. *Journal of Applied Microbiology,* 111: 1148-1158.

Zhu, Y., Nishino, N., Kishida, Y. and Uchida, S. 1999. Ensiling characteristics and ruminal degradation of Italian ryegrass and lucerne silages treated with cell wall-degrading enzymes. *Journal of the Science of Food and Agriculture,* 79: 1987-1992.

Animal Nutrition and Feed Technology (2013) 13: 507-516

Chemical Composition and *In Vitro* Digestibility of *Pleurotus ostreatus* Spent Rice Straw[#]

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ABSTRACT

Khattab, H.M., Gado, H.M., Salem, A.Z.M., Camacho, L.M., El-Sayed, M.M., Kholif, A.M., El-Shewy, A.A. and Kholif, A.E. 2013. Chemical composition and in vitro digestibility of Pleurotus ostreatus spent rice straw. Animal Nutrition and Feed Technology, 13: 507-516.

The nutritive value of rice straw (RS) and *Pleurotus ostreatus* spent rice straw (SRS) was studied by analyzing its proximate composition, fiber fractions, *in vitro* digestibility, amino acids content and scanning electron microscopy (SEM). The possibility of replacing berseem clover (BC; *Trifolium alexandrinum*) with SRS at different levels also was studied. Results showed higher protein content for SRS compared to RS (3.4 to 11.7%) while, DM, OM, NFE, CF, NDF, ADF, ADL, hemicellulose and cellulose were less for SRS than for RS. Highest concentration of amino acids (mg/100 g) was in SRS compared to RS. The SEM showed an extensive damage of SRS when compared to RS. Data also showed that SRS had higher *in vitro* dry matter disappearance (DMD) and *in vitro* organic matter disappearance (OMD) compared to RS. Results of *in vitro* study also, indicated that the levels of 50 and 90% replacement had the highest values of DMD and OMD compared to the other levels. It could be concluded from this study that treatment of RS with *Pleurotus ostreatus* improved the potential feeding value of the resultant substrates (i.e. SRS) as feed resources for ruminants and possibility of replacing BC with SRS at high levels of up to 50 or 90% from diets.

Key words: Biodegradation, *Pleurotus ostreatus*, Rice straw, Spent rice straw.

INTRODUCTION

Lignocellulosic biomass is not only a renewable resource but also it is the richest abundant source of organic components on the earth (Taniguchi *et al.,* 2005). Rice

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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cultivation is one of the most important agriculture practices worldwide. However, it produces large amounts of rice straw (RS) by-products. The FAO has estimated RS production at about 723 million tons annually (FAO, 2013). Most of RS is burnt in the field as a way to clear the field and get rid of disease from previous crop. This practice raises the problems of increasing CO_2 emission which leads to increase global warming.

The use of RS and other lignocellulosic materials as animal feed is limited by their low nutritive value and low nitrogen content (Jalc *et al.,* 1996). Various problems associated with the practical utilization of these materials have not yet been solved. One of the key problems hindering the effective utilization of these resources is the low susceptibility of lignocellulose to hydrolysis, mainly attributable to the crystalline structure of the cellulose fibrils surrounded by hemicellulose, and by the presence of the lignin seal which prevents enzymes penetration (Chahal and Chahal, 1999). Intensive researches and developmental studies on the effective utilization of lignocellulosic materials have been done (Taniguchi *et al.,* 2005). Biological delignification of straws by white-rot fungi seems to be a promising way for improving their nutritive value (Fazaeli *et al.,* 2002).

In recent years, the significance of amino acids has been realized, not only in terms of their nutritional availability for anabolic use but also the fact that they are involved in a number of metabolic pathways. It serves in important functions including protein and energy metabolism, gluconeogenesis, lipid-fatty acid metabolism, and in mammary synthesis of milk protein and lactose (El-Kadi *et al.,* 2006; Chalamcherla *et al.,* 2010). Defining and quantifying amino acid requirements will become an important consideration in the next generation of feeding schemes for dairy cattle beyond the current emphasis on identification of limiting amino acids (Chalamcherla *et al.,* 2010).

The objectives of this work were to study the chemical and histological changes occurred as a result of *Pleurotus ostreatus* fungi cultivation on RS and nutritional evaluation of replacing Egyptian berseem clover (BC; *Trifolium alexandrinum*) with the spent rice straw (SRS), the product after *Pleurotus ostreatus* cultivation and harvest.

MATERIALS AND METHODS

Mushroom cultivation process was carried out at a private mushroom farm, *Ploshia* for mushroom production, Cairo, Egypt. The technique was carried out according to the method described by (Oei, 2005).

Spent rice straw preparation

Clean and dried RS was obtained from the field, weighed and soaked overnight for moisture absorption, then let stand for 15 min in order that the excess water can drain off. About 5% calcium carbonate ($CaCO_3$) on DM basis was added to maintain the neutral pH (7.0). The prepared substrates were sterilized by hot water (100°C) for one hour in order to kill competing micro-organisms, and to prevent pests and diseases. After the sterilization, the substrates were put into a clean plastic sheet for draining and

cooling. As soon as the substrates were cooled down to 20-25°C and drained, they were put into the plastic bags (25 cm wide and 40 cm height). Good quality spawns of Oyster mushrooms (*Pleurotus ostreatus*) were obtained from Mushroom Laboratory, Food Industries Department, National Research Centre, Egypt to inoculate the straw in the plastic bags at about 3 to 8% of the weight of the substrate.

The bags were tightly closed and pin holes were made on the bags to ensure that enough oxygen can reach the substrate. The bags were subsequently kept in a spawn running room at 25°C under dark condition until mycelium was formed. After mycelium formation (after about 21 d), large holes were made in the polythene bags to allow the normal development of fruit bodies. The bags were then put in the growing rooms containing shelves at 22°C with a 12 h photoperiod (1500-2000 Lux) and 85-90% relative humidity. Adequate ventilation was provided to prevent increase of CO_2 concentration. The bags were collected after seven weeks when the mushroom fruits were harvested two times and dried under the sun.

In vitro studies

Two laboratory *in vitro* trials were conducted. The first one aimed to compare *in vitro* dry matter disappearance (DMD) and *in vitro* organic matter disappearance (OMD) of both RS and *Pleurotus ostreatus* SRS. The second study aimed to compare different replacement levels of BC with SRS. In the first study, 12 incubation flasks (250 ml volume) were used (6 flasks per each treatment) while, the second study used 72 incubation flasks (6 flasks per each treatment in addition to 6 flasks as blank) to compare OMD and DMD of 11 replacement levels 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% of BC with SRS. The procedures of the *in vitro* techniques were carried out according to Fondevila and Pérez-Espés (2008). Flasks filled with 140 ml of incubation solution prepared under a CO_2 atmosphere, including a buffer solution, macromineral and micromineral solutions, a reduction solution and rumen inoculum obtained from Baladi bucks fed on good quality alfalfa hay using stomach tube. Flasks were sealed and maintained at 38°C in a shaking water bath (20 oscillations/min) for 48 h.

Chemical analysis

Dried ingredients samples were ground through a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) using a 1 mm screen. Samples were analysed for dry matter (DM, method ID 930.15), crude protein (CP, method ID 954.01), ether extract (EE, method ID 920.39), crude fiber (CF, method ID 962.09) and ash (method ID 942.05) according to AOAC (1995). Fiber fractionations were done according to Goering and Van Soest (1970) and Van Soest *et al*. (1991). Organic matter (OM) was calculated.

Determination of amino acids (HCl - hydrolyzed)

Determination of individual amino acids was performed using dried samples of RS and SRS. The procedure was performed according to the method described by (Bailey, 1967) using Eppendorff LC3000 (Germany) amino acid analyser.

Scanning electron microscopy

Scanning electron microscopy (SEM) of RS and SRS was performed according to the method described by Liu *et al*. (2005) using a scanning electron microscope (JSM-6400; JEOL, Tokyo).

Calculations and statistical analysis

Analysis of variance in a completely randomize design using general linear model procedure and Duncan test at 5% level of significance were used to compare the results for the different replacing levels using software SAS/STAT® (SAS, 2001, Version 8.02, SAS Institute Inc., Cary, NC, USA).

RESULTS

The cultivation of *Pleurotus ostreatus* on RS increased its content from CP, EE, ash and silica, while cultivation process decreased RS content of CF, DM, OM, NFE, NDF, ADF, ADL, hemicellulose and cellulose than RS (Table 1).

Table 1: Chemical composition and *in vitro* digestibility of rice straw and spent rice straw (% of DM basis)

	Rice straw	Spent rice straw
Chemical composition		
DM	93.1	83.1
OM	83.9	70. 9
CP	3.4	11.7
CF	35.6	24.0
EE	1.6	2.7
Ash	16.1	29.1
Silica	11.7	18.5
NDF	63.5	39.6
ADF	36.2	30.2
ADL	9.4	4.3
Hemicellulose	27.24	9.42
Cellulose	26.87	25.91
In vitro digestibility (%)		
DMD (±SE)	50.8^{b}±0.5	60.4^{a}±2.5
OMD (±SE)	54.4±2.5	60.6±5.5

Each value is a mean of 6 samples

NDF, Neutral detergent fibers; ADF, Acid detergent fibers; ADL, acid detergent lignin

^{ab}Means with different superscripts in the same column are significant ($P<0.05$)

Total amino acids concentration (mg/100 g) was increased in SRS (8151) than in RS (5951) due to the cultivation of *Pleurotus ostreatus*. Only three individual amino acids were lowered in SRS; leucine, tyrosine and phenylalanine, while the methionine and lysine concentrations were higher in SRS than in RS (Table 2).

Table 2. Amino acids content (mg/100 g) of rice straw and spent rice straw

Amino acid	Rice straw	Spent rice straw
Essential amino acid		
Threonine	202	387
Methionine	42	54
Phenylalanine	496	208
Histidine	220	567
Lysine	190	440
Non- essential amino acids		
Serine	254	576
Glutamic acid	1133	1382
Alanine	524	890
Tyrosine	526	99
Arginine	217	569
Aspartic	521	858
Branched chain amino acids		
Leucine	691	601
Isoleucine	196	221
Glycine	130	341
Valine	250	312
Proline	359	646
Total	5951	8151

The surface of the SRS tissues (Fig. 1B and 1D) seemed fragile, while the papillae, wart-like structures and micro-hairs were nearly disappeared and the cuticle wax silica layer was partly dissolved to clearly show the short-cells (Fig 1A and 1C). Moreover, an extensive damages of RS tissues by the fungus *Pleurotus ostreatus* on the surface of SRS when compared with RS.

Table 3. *In vitro* dry matter disappearance (DMD) and *in vitro* organic matter disappearance (OMD) at different levels of replacement of berseem clover with spent rice straw

Level of replacement (%)	Disappearance (%)	
	DMD	OMD
0	$56.2^{b}\pm1.2$	$66.7^{ab}\pm1.3$
10	$56.2^{b}\pm2.0$	$63.9^{b}\pm1.6$
20	$57.2^{ab}\pm2.2$	$65.7^{ab}\pm1.6$
30	$57.4^{b}\pm2.9$	$65.4^{ab}\pm1.5$
40	$58.1^{ab}\pm1.5$	$64.3^{ab}\pm1.3$
50	$60.7^{ab}\pm1.8$	$69.\ 6^{a}\pm1.5$
60	$59.4^{ab}\pm1.6$	$66.4^{ab}\pm1.4$
70	$59.9^{ab}\pm2.5$	$65.6^{ab}\pm2.0$
80	$60.3^{b}\pm3.3$	$67.7^{ab}\pm2.8$
90	$62.3^{a}\pm3.2$	$70.9^{a}\pm2.7$
100	$56.5^{b}\pm1.9$	$59.9^{c}\pm1.1$

Each value is a mean of 6 samples

[abc]Means with different superscripts in the same column are significant ($P<0.05$).

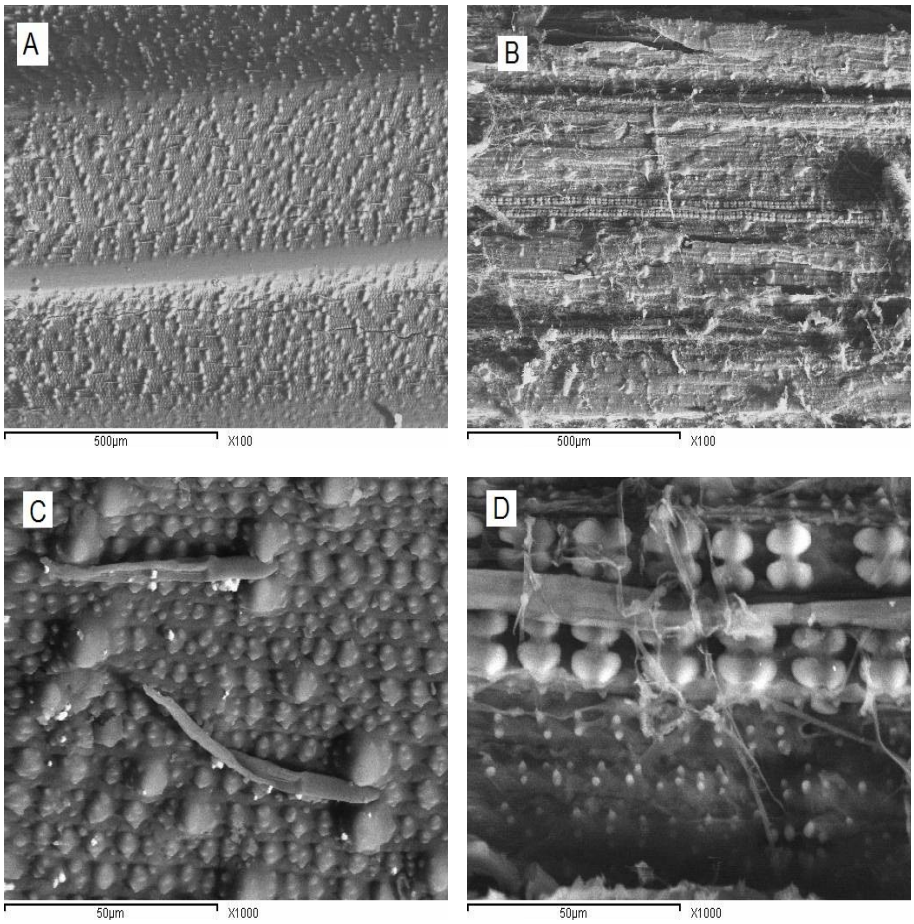

Fig. 1: Scanning electron microscope of rice straw and *Pleurotus ostreatus* spent rice straw- a) Rice straw (*100X*); b) *Pleurotus ostreatus* SRS (*100X*); c) Rice straw (*1000X*); d) *Pleurotus ostreatus* SRS (*1000X*)

Results of the first experiment showed that *Pleurotus ostreatus* SRS had higher ($P < 0.05$) DMD and OMD (Table 1) compared to RS.

Results of the second *in vitro* experiment indicated that the levels of 50 and 90% replacement increased values of DMD and OMD compared with the other levels. The lowest values of DMD, OMD were observed with 10, and 100% replacement levels compared with the other levels. On the contrary, the addition of SRS almost lowered OMD compared with 0% replacement level (Table 3).

DISCUSSION

Chemical composition and cell wall constituents

Fungus obtains their requirements from decaying the OM, in particular, the lingo cellulolytic constituents. This finding could explain the changes resulted from *Pleurotus* cultivation on RS. *Pleurotus ostreatus* belongings to the basidomycetes which produce enzymes such as lignin peroxidase, manganese peroxidase, H_2O_2 producer enzymes, arylachol oxidase and laccase. These fungi are unable to supply all their carbon and energy requirements from lignin, and therefore require substrates such as cellulose or other carbon sources for their growth and delignification (Ruggeri and Sassi, 2003). They consume of cellulose and hemicellulose with the growth of *Pleurotus ostreatus* on RS and thus decreased OM and ash contents in SRS. Delignification of RS probably increases hemicellulose solubility, but cellulose remains insoluble and its contents changes less than hemicellulose (Jafari *et al.,* 2007). The higher CP content in SRS compared with RS may be due to one of the following reasons: the presence of micro-organisms, extracellular enzymes and residual media ingredients (*i.e.* mycelium) in SRS (Ball and Jackson, 1995; Siddhant and Singh, 2009); the capture of access N by aerobic fermentation by fungus (Akinfemi, 2010); the proliferation of fungi during degradation (Akinfemi and Ogunwole, 2012).

Such results were obtained by Jafari *et al.* (2007), Akinfemi (2010) and Akinfemi and Ogunwole (2012) when *Pleurotus ostreatus* cultivated on RS causing an increase of CP, ash content and decrease in the content of hemicelluloses, OM, CF, ADF, NDF and ADL.

Amino acid content

Comparing amino acids content before and after cultivation of *Pleurotus ostreatus* on RS showed an increase in the content of total amino acids which associated with the presence of fungus mycelium and mushroom bodies by-products. The improvement in the total amino acids content of the RS was the result of an increased in the quantity of different amino acids. However, the increase in the quantity was not uniform for all the amino acids.

Histological changes of RS tissues

Epidermis is an important protective tissue in plants due to its wax and silica layers, and it may decrease water loss from evaporation and defend against bacterial and fungal pathogens. However, it becomes an obstacle to degradation by rumen micro-organisms (Wang *et al.,* 2007). The morphological changes induced by *Pleurotus ostreatus* were examined by SEM to obtain insight into the structural modification in the RS. An extensive damage was observed in RS tissues treated with *Pleurotus ostreatus* (i.e., SRS) when compared with untreated RS, suggesting the efficacy of *Pleurotus ostreatus* to produce lignocellulolytic enzymes and degrade of lignocellulose in RS.

Photos of SEM emphasized that the cultivation of *Pleurotus ostreatus* resulted in an increase in susceptibility of RS to enzymatic hydrolysis due to partial degradation of the lignin which is responsible for preventing penetration of cellulase in the RS. Based on histological changes of stem epidermis, it may be suggested that cultivation of *Pleurotus ostreatus* affect epidermis of straw stem differently, and result in different degradability of epidermis.

In vitro digestibility

Increased DMD in SRS may be due to the decreased CF content and different fiber fractions due to enzymes action, and also due to the increased CP content of SRS (Jafari *et al.,* 2007). Likewise, delignification results in changes in cell wall structure beyond the simple removal of lignin and cell constituents are readily available for rumen micro-organisms (Mirzaei *et al.*, 2007) and this could lead to increase RS digestibility. However, some researchers reported that fungal delignification causes low digestibility values (Jalc *et al.,* 1996). This difference in reported results may be possibly related to many factors such as silica content of RS and fungus growth stage influence cell wall degradation and their digestibility. Silica has shown to exert an anti-microbial effect on the rumen bacteria, inhibit cellulose and cellulolytic microbes and thus reduce digestion of plant cell wall. However, this effect depends on solubilization of silica (Bae *et al.,* 1997). However, increased digestibility in SRS, in spite of higher silica content, might be related to solubilization of silica.

The presences of highly lignified tissues in RS makes a physical barrier and prevents the accessibility of highly digestible tissues to the action of hydrolytic enzyme for rumen micro-organisms (Karunanandaa *et al.,* 1995) and increased digestibility associated with the degradation of structural carbohydrates (Mukherjee and Nandi, 2004). Results reported here are in agreement with many other findings (Ko *et al.,* 2005; Jafari *et al.,* 2007).

Replacing BC with SRS

Increased DMD and OMD with both 50 and 90% replacement levels compared with the other levels may be related with the enhancement of structure occurred with SRS compared with RS. This was related with the delignification and hydrolysis occurred with the extracellular enzymes secreted from the fungus. The addition of SRS decreased OMD compared with 0% replacement level which may be due to the high content of ash in SRS. The high content of ash shown to exert an anti-microbial effect on the rumen microflora which inhibits digestion. The complete replacement of BC with SRS may increase the level of phenolic compounds which resulted from lignin decomposition which exert negative effects on media microflora.

CONCLUSION

Results suggest that *Pleurotus ostreatus* is a suitable fungus for improving the nutritive value of RS as a ruminant feed not only by improving the chemical composition but also enhancing the digestibility. *Pleurotus ostreatus* has a good potential as feed stuff for ruminant animals and could be used in combination with other feedstuffs. However, further work may be required involving its validation under *in vivo* conditions.

REFERENCES

AOAC. 1995. *Official Methods of Analysis*, 16th ed. Association of Official Analytical Chemists, Washington, DC., USA.

Akinfemi, A. and Ogunwole, O.A. 2012. Chemical Composition and *in vitro* digestibility of rice straw treated with *Pleurotus ostreatus, Pleurotus Pulmonarius* and *Pleurotus tuber-regium*. *Slovak Journal of Animal Science,* 45: 14-20

Akinfemi, A. 2010. Bioconversion of peanut husk with white rot fungi: *Pleurotus ostreatus* and *Pleurotus pulmonarius*. *Livestock Research for Rural Development*. *Volume 22, Article # 49*. Retrieved July 24, 2013, from http: //www.lrrd.org/lrrd22/3/akin22049.htm

Bae, H.D., MacAllister, T.A., Kokko, E.G., Leggett, F.L. and Cheng, K.J. 1997. Effect of silica on colonization of rice straw by ruminal bacteria. *Animal Feed Science and Technology,* 65: 165-181.

Bailey, J.L. 1967. *Techniques in Protein Chemistry*. Elsevier Publishing Co., London, UK.

Ball, A.S. and Jackson, A.M. 1995. The recovery of lignocellulose degrading enzymes from spent mushroom compost. *Bioresource Technology*, 54: 311-314.

Chahal, P.S. and Chahal, D.S. 1999. Lignocellulose waste: biological conversion. In: *Bioconversion of Waste Materials to Industrial Products,* 2nd ed., (Ed. A.M. Martin). Blackie Academic and Professional, London, pp. 376-422.

Chalamcherla, V.L., Singaracharya, M.A. and Vijaya, L.M. 2010. Amino acids profile of the lignocellulosic feed treated with cellulase-free lignolytic mutants of *Pleurotus ostreatus*. *Bio Resources,* 5: 259-267.

El-Kadi, S.W., Baldwin, V.I., Sunny, R.L., Owens, S.L. and Bequette, B.J. 2006. Intestinal protein supply alters amino acid, but not glucose metabolism by the sheep gastro intestinal tract. *Journal of Nutrition*, 136: 1261-1269.

FAO, 2013. Food and Agriculture Organization of the United Nations. *Production Yearbook*. FAO Production Yearbook, Rome, Italy.

Fazaeli, H., Jelan, Z.A., Azizi, A., Rouzbehan, Y., Mirhady, S.A. and Liang, J.B. 2002. Biodegradation of wheat straw by *Pleurotus* fungi: The growth rate on untreated and urea treated wheat straw. *Malaysian Journal of Animal Science,* 7: 39-49.

Fondevila, M. and Pérez-Espés, B. 2008. A new *in vitro* system to study the effect of liquid phase turnover and pH on microbial fermentation of concentrate diets for ruminants. *Animal Feed Science and Technology,* 144: 196-211

Goering, H.K. and Van Soest, P.J. 1970. *Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications)*, Agricultural Handbook No. 379. US Agriculture Research Service, Washington, DC.

Jafari, M.A., Nikkhah, A., Sadeghi, A.A. and Chamani, M. 2007. The effect of *Pleurotus* spp. fungi on chemical composition and *in vitro* digestibility of rice straw. *Pakistan. Journal of Biological Science,* 10: 2460-2464.

Jalc, D., Nerud, F., Erbanová, P. and Siroka, P. 1996. Effect of white-rot basidiomycetes-treated wheat straw on rumen fermentation in artificial rumen. *Reproduction, Nutrition and Development*, 36: 263-270.

Karunanandaa, K., Varga, G.A., Akin, D.E., Rigsby, L.L. and Royse, D.J. 1995. Botanical fractions of rice straw colonized by white-rot fungi: Changes in chemical composition and structure. *Animal Feed Science and Technology,* 55: 179-199.

Ko, H.G., Park, H.G., Park, S.H., Choi, C.W., Kim, S.H. and Park, W.M. 2005. Comparative study of mycelial growth and basidiomate formation in seven different species of edible mushroom genus *Hericium*. *Bioresource Technology*, 96: 1439-1444.

Liu, D., Liu, J.X., Zhu, S.L., Chen, X.J. and Wu, Y.M. 2005. Histological investigation of tissues and cell wall of rice straw influenced by pretreatment with different chemicals and rumen degradation. *Journal of Animal and Feed Sciences,* 14: 373-387.

Mirzaei, J., Tabari, M. and Daroodi, H. 2007. The Effect of *Pleurotus* spp. Fungi on Chemical Composition and *in vitro* Digestibility of Rice Straw. *Pakistan. Journal of Biological Science,* 10: 2460-2464.

Mukherjee, R. and Nandi, B. 2004. Improvement of *in vitro* digestibility through biological treatment of water hyacinth biomass by two *Pleurotus* species. International *Biodeterioration and Biodegradation,* 53: 7-12.

Oei, P. 2005. *Small Scale Mushroom Cultivation*. Didigrafi, Wageningen, The Organization of the United Nation, Rome, pp. 37

Ruggeri, B. and Sassi, G. 2003. Experimental sensitivity analysis of trickle bed bioreactor for lignin peroxidases production by *Phanerochaete chrysosporium*. *Process Biochemistry,* 38: 669-1676.

SAS. 2001. *Statistical Analysis System (SAS) User's Guide*. SAS Institute Inc., North Carolina, USA.

Siddhant, C. and Singh, S. 2009. Recycling of spent oyster mushroom substrate to recover additional value. *Kathmandu University Journal of Science,* 5: 66-71.

Taniguchi, M., Suzuki, H., Hoshino, K., Watanabe, D. and Tanaka, T. 2005. Evaluation of Pretreatment with *Pleurotus ostreatus* for Enzymatic Hydrolysis of Rice Straw. *The Journal of Bioscience and Bioengineering,* 100: 637-643.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3597.

Wang, J.K., Liu, J.X., Li, J.Y., Wu, Y.M. and Ye, J.A. 2007. Histological and rumen degradation changes of rice straw stem epidermis as influenced by chemical pretreatment. *Animal Feed Science and Technology,* 136: 51-62.

Animal Nutrition and Feed Technology (2013) 13: 517-525

Effect of Treatment with Fibrolytic Enzymes or Ammonia on the Nutritive Value of Guineagrass (*Panicun maximum*) Hay[#]

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ABSTRACT

Dean, D.B., Valencia, E., Krueger, N.A. and Adesogan, A.T. 2013. Effect of treatment with fibrolytic enzymes or ammonia on the nutritive value of guineagrass (Panicum maximum) hay. Animal Nutrition and Feed Technology, 13: 517-525.

This study evaluated the effect of applying four fibrolytic enzymes or NH_3 on the nutritive value of 6- (6-wk) and 8-week (8-wk) regrowths of guineagrass (*Panicum maximum*) hay. The NH_3 was applied at 4% DM and enzymes Promote (Pr), Biocellulase X-20 (X-20), Fibrozyme (Fib) and Biocellulase A-20 (A-20) were applied at 0, 0.5x, 1x and 2x manufacturer-recommended rates to 2 kg of guineagrass hay in triplicate. Hays were stored for 3-weeks before determination of chemical composition and 6 and 48 h *in vitro* digestibility of DM (IVDMD), NDF (IVNDFD), and ADF (IVADFD). Crude protein concentration, 6 and 48 h IVDMD, and 48 h IVNDFD and IVADFD values were greater ($P<0.01$), while ADF and NDF concentrations were lower ($P<0.01$) in 6-wk hays compared to 8-wk hays. Additive treatment did not affect ADF or NDF concentration of 6-wk hays, but in 8-wk hays, X20 and Fib treatment reduced NDF and ADF concentrations (additive x maturity interaction, $P<0.1$). Ammoniation increased ($P<0.01$) CP concentration of 6 and 8-wk hays by 42 and 91%, respectively (additive x maturity interaction, $P<0.01$). Ammoniation increased ($P<0.01$) 6 h IVDMD by 35% and increased ($P<0.01$) 48 h IVDMD and IVNDFD by 10%, and there were no maturity x additive interactions for these measures. Additive treatment did not increase 48 h IVADFD. This study shows that guineagrass quality decreases as the duration between harvests increases. Certain enzymes produced small (<4%) decreases in the fiber concentration of 8-wk hays, but enzymes had no other effects on nutritive value. Ammoniation increased CP concentration and *in vitro* DM and NDF digestibility, therefore ammoniation was the only treatment that increased the nutritive value of guineagrass hays.

Key words: Guineagrass hay, Enzymes, Ammonia, Nutritive value.

INTRODUCTION

Forages represent the most important, cost effective feed resource in ruminant nutrition (Jung and Allen, 1995). However, the relatively low quality of tropical forages

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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militates against their use as the sole feed for actively growing or high-performing ruminants. Several attempts have been made to improve forage quality genetically or by chemical or biological treatments. One of the most important goals in this regard is to improve the fiber digestibility of the forages. Some chemical and biological treatments have been effective at achieving this objective.

Ammoniation is one of the most studied chemical treatments for enhancing fiber digestibility and several reports have described its' effectiveness for improving both forage quality and animal performance. Ammoniation increases forage crude protein (CP) concentration and substantially reduces the concentration of neutral detergent fiber (NDF) in forages. Most of the loss of NDF is due to hydrolysis of hemicellulose, though the disruption of chemical linkages between lignin and hemicellulose also occurs (Weiss and Underwood, 1995; Barrios-Urdaneta and Ventura, 2002). Additional benefits of ammoniation include reduced yeast and mold growth, and less aerobic deterioration of high-moisture hay and silage (Woolford and Tetlow, 1984; Bates *et al.*, 1989). Consequently, feeding ammoniated forage often results in increased daily gain and dry matter (DM) intake in beef cattle (Vagnoni *et al.*, 1995; Brown and Pate, 1997).

Fibrolytic enzyme application is one of the most studied biological treatments for improving forage quality and animal performance. Such enzymes have been effective at improving the utilization of a wide range of diets containing roughages (Rode and Beauchemin, 1998) due to improved fiber hydrolysis (Colombatto *et al.*, 2003) which often results in increased digestibility (Christensen, 1997; Rode *et al.*, 1999) and voluntary intake (Pinos-Rodriguez *et al.*, 2002). Nevertheless, another study has shown that exogenous enzymes did not consistently improve forage utilization by ruminants (Lewis *et al.*, 1999). This inconsistency is attributable to several factors such as differences in enzyme type and activity, treatment duration, application method, diet composition and level of animal performance. The aim of this experiment was to evaluate the effect of ammonia and proprietary fibrolytic enzyme application on the nutritive value of guineagrass hay, harvested at different maturity stages.

MATERIALS AND METHODS

Enzyme and ammonia application

The effect of applying NH_3 or four fibrolytic enzyme complexes (Promote®, Pr, Cargill, Minnetonka, MN), Biocellulase X-20® (X-20, LodeStar, IL, USA), Fibrozyme® (Alltech, Lexington, KY, USA) and Biocellulase A-20® (A-20) (LodeStar, IL, USA) was measured on the chemical composition and *in vitro* and digestibility of guineagrass (*Panicun maximum*) hays, harvested at 6 and 8-wk regrowth. The ammonia was applied at 40 g/kg DM and the enzymes were applied at 0 (Control), 0.5, 1 and 2 times the rates recommended by the respective manufacturers. This was done because the optimal application rate for C_4 grass hays was unknown. The actual application rates

are shown in Table 1. The enzymes were dissolved in 500 ml of water and applied in a fine spray to 3 replicates of 2 kg of each hay. Treated hays were stored for 3-weeks in plastic bags and then chemically characterized. The manufacturer-stipulated activities of the enzymes are shown in Table 2. Cellulase activity was also determined at 39°C and pH 5.5 using the filter paper method (Wood and Bhat, 1988) and the values obtained for Pr, X-20, and A-20 were 33.7, 22, and 51.3 filter paper units/g, respectively, where one unit of activity is the amount of enzyme that releases exactly 2 mg of glucose from 50 g of filter paper in 60 min. Xylanase activity was determined at 39°C and pH 5.5 using the di-nitro salicylic acid procedure (Bailey *et al.*, 1992) and the values obtained for Pr, X-20, and A-20 were 5190, 7025, and 3530 µmol of xylose released/min/ml, respectively.

Table 1. Actual enzyme application rates used

Enzyme	Application rate		
	0.5x	1x	2x
Promote[†] (mg/kg DM)	650	1300	2600
Biocellulase X-20[‡] (mg/kg DM)	7.3	14.5	29
Biocellulase A-20[‡] (mg/kg DM)	7.3	14.5	29
Fybrozyme[§] (mg/kg DM)	89	178	356

[†]Cargill, Minnentoka, MN; [‡]LodeStar, Channahon, IL, USA; [§]Alltech, Lexington, KY, USA.

Table 2. Manufacturer-stipulated enzyme activities

Enzyme	Enzymatic activity			
	Cellulase (Units/g)	Xylanase (Units/g)	B-Glucanase (Units/g)	Amylase (Units/g)
Promote[†]	1,200	-	-	-
Biocellulase X-20[‡]	5,700	16,000	600	1,200
Biocellulase A-20[‡]	6,000	400	4,300	3,100

[†]Cargill, Minnentoka, MN, USA; [‡]LodeStar, Channahon, IL, USA.

Laboratory analysis

The NDF and ADF concentrations (Van Soest *et al.*, 1991) of the samples and digested residues were determined without amylase pretreatment using an ANKOM^{200} fiber analyzer (ANKOM Technology, Macedon, NY). Hemicellulose was calculated by difference from NDF and ADF concentrations. Water-soluble carbohydrates (WSC) were determined with the anthrone reaction assay (Ministry of Agriculture Fisheries and Food, 1986). Crude protein (CP) was determined by digesting 0.5 g of sample using a micro Kjeldahl apparatus (Labconco Corporation, Kansas City, MO) and the N concentration was determined (Noel and Hambleton, 1976) using a Technicon Auto Analyzer (Technicon, Tarrytown, NY, USA).

The *in vitro* digestibility of DM (IVDMD), NDF (IVNDFD) and ADF (IVADFD) were determined in duplicate runs after incubating forage samples in buffered rumen fluid for 48-h using two ANKOM^{II} Daisy Incubators (ANKOM

Technology, Macedon, NY). The buffer was prepared according to the ANKOM Technology procedure. The rumen fluid was obtained before feeding from two, non-lactating, fistulated cows, fed 9 kg of Coastal bermudagrass hay and 400 g of soybean (*Glycine max*) meal daily.

Statistical analysis

A completely randomized design with 3 replicates per treatment was used to quantify the effects of enzyme or NH_3 application on chemical components and *in vitro* digestibility.

The model used was:

Yijk: μ + Ti + Ei

Where, Yij: dependent variable; μ: general mean; Ti: enzyme and NH_3 effect; Ei: experimental error.

Data were analysed using the GLM procedure of SAS (1995). Orthogonal contrasts were used to compare additive treatment means, and polynomial contrasts were used to determine the effect (linear, quadratic and cubic) of increasing the amount of enzyme application.

RESULTS AND DISCUSSION

Effect of fibrolytic enzymes and ammonia on chemical composition

Table 3 shows low CP and high NDF and ADF concentrations in the 6-wk and 8-wk regrowth guineagrass hays which are typical of tropical grasses. These values agree with those obtained by Jung and Allen (1995) who concluded, that depending on the stage of maturity, cell walls represent between 30 and 80% of plant DM in grasses so that under most circumstances, the bulk of carbohydrates in mature grasses are from cell wall polysaccharides. According to Aganga and Tshwenyane (2004) grass quality parameters decreased from the young to mature stages as a result of differences in plant composition between levels of maturity. The presence of an increased proportion of plant stems, typical of older plants, may restrict access to leafy parts and force animals to consume lower quality herbage.

The NDF and ADF concentrations at both maturities were higher than those reported by Ngo and Wiktorsson (2003) for 4, 6, 8 and 10-wk regrowths of guineagrass hays and than those obtained by Aganga and Tshwenyane (2004) in hays of different cultivars of guineagrass. The CP concentration of enzyme-treated hays was similar to that obtained by Bamikole *et al.* (2004) in 6-wk regrowth unfertilized guineagrass hay, but lower than those reported by Aganga and Tshwenyane (2004) in hays of different cultivars of guineagrass at 6 or 8-wk regrowth.

The NDF concentration of guineagrass hays at 6-wk regrowth was unaffected by ammonia or enzyme treatments (Table 3). However, Pr (linear, $P<0.01$) and Fib (linear, $P<0.05$) decreased the NDF concentration as the application rate of both

enzymes increased (Table 4). For the 8-wk regrowth, X-20 and Fibrozyme decreased ($P<0.01$) the concentration of this fraction, compared to the control hay. None of the enzymes produced polynomial effects, which indicates that even at the lower application rate, the above mentioned enzymes were able to hydrolyze the NDF fraction of the hays (Table 4). Analysis across both maturities did not show effects of enzyme treatment on NDF concentration. Results for the 8-wk regrowth partly agree with those of Colombatto *et al*. (2003) and Hristov *et al*. (1998), who observed that the NDF fraction was hydrolyzed into sugars, however, disagree with those of Brown (1993), who observed that ammoniation decreased ($P<0.01$) the NDF concentration of stargrass (*Cynodon nlemfuensis*) hay.

Table 3. Effect of enzyme type and ammonia treatment on chemical composition of guineagrass at different stages of maturity

Additive		Variable, g/kg DM								
		NDF			ADF			CP		
		6-wk	8-wk	Mean	6-wk	8-wk	Mean	6-wk	8-wk	Mean
NH_3		790	852 ^{bc}	821^{a}	485	557^{b}	521^{b}	93^{a}	47^{a}	70^{b}
Control		812	865 ^{b}	839^{b}	470	559 ^{b}	515^{b}	69 ^{b}	23^{b}	46^{a}
Pr	0.5x	816	865		480	553		66	26	
	1x	796	872		480	557		65	22	
	2x	774	853		468	550		68	23	
	Mean	795	863 ^{b}	829^{b}	476	553^{bc}	515^{b}	67 ^{b}	24 ^{b}	45^{a}
	Level effect	C*	NS		NS	NS		NS	C*	
X-20	0.5x	805	854		477	550		59	25	
	1x	809	840		482	546		64	26	
	2x	818	833		494	545		64	25	
	Mean	811	842^{ac}	826^{b}	485	547^{ac}	515^{b}	62 ^{b}	25 ^{b}	44^{a}
	Level effect	NS	NS		NS	NS		NS	NS	
Fib	0.5x	815	839		484	545		70	26	
	1x	798	829		474	538		65	25	
	2x	793	840		468	532		63	26	
	Mean	801	835^{a}	819^{a}	475	538^{a}		66 ^{b}	26^{b}	45^{a}
	Level effect	L*	NS		NS	NS		L*	NS	
A-20	0.5x	787	872		459	547		63	26	
	1x	794	870		463	545		66	23	
	2x	801	867		479	534		64	26	
	Mean	794	869 ^{b}	831^{b}	467	542^{a}	504^{a}	64 ^{b}	25 ^{b}	45^{a}
	Level effect	NS	NS		C*	NS		NS	Q*	
SEM	4.37	4.18			4.37	3.38		1.14	0.62	
Effect of P values										
Additive		0.14	<0.01	0.306	0.09	<0.01	<0.01	<0.01	<0.01	<0.01
Maturity			<0.01			<0.01			<0.01	
Addit*Matur			<0.01			0.096			<0.01	

NS- Not significant, $P>0.05$; *$P<0.05$; L- Linear, Q- Quadratic, C- Cubic;

^{a,b,c}Means in the same column with different superscripts differed; Addit=Additive; Matur= Maturity.

Table 4. Effect of enzyme application rates on *in vitro* digestibility (g/kg DM) of different fractions of guineagrass hays.

Additive		*In vitro* digestibility, g/kg DM								
		DM			NDF			ADF		
		6-wk	8-wk	Mean	6-wk	8-wk	Mean	6-wk	8-wk	Mean
NH_3		491 [a]	392[a]	442[a]	460[a]	355[a]	408[a]	436[a]	303[a]	369[a]
Control		474[a]	316[b]	395[b]	442[a]	278[bc]	360[b]	399[a]	218[b]	308[ab]
Pr	0.5x	475	329		422	297		404	248	
	1x	463	348		422	315		439	284	
	2x	463	327		380	294		388	260	
	Mean	461[a]	335[b]	398[b]	408[a]	302[b]	355[b]	411[a]	264[ac]	337[a]
	Level effect	NS	NS	NS	NS	NS	NS	NS	NS	NS
X-20	0.5x	433	342		399	300		374	255	
	1x	380	327		355	268		398	234	
	2x	430	306		346	249		358	228	
	Mean	414[b]	325[b]	370c	374[b]	272[c]	323[c]	360[b]	239[b]	299[b]
	Level effect	NS	NS	NS	NS	NS	NS	NS	NS	NS
Fib	0.5x	459	309		397	252		369	221	
	1x	442	313		399	241		363	193	
	2x	443	323		371	302		347	224	
	Mean	448[a]	353[b]	382[bc]	389[b]	265[c]	327[c]	360[b]	213[b]	286[b]
	Level effect	NS	NS	NS	NS	NS	NS	NS	NS	NS
A-20	0.5x	438	330		379	313		333	248	
	1x	424	315		384	291		332	229	
	2x	432	347		397	323		364	260	
	Mean	431[a]	330[b]	381[bc]	387[b]	309[b]	348[b]	343[b]	245[bc]	294[b]
	Level effect	NS	NS	NS	NS	NS	NS	NS	NS	NS
SEM		11.70	7.49		12.85	11.03		12.94	10.39	
Effect of P values										
Additive		0.011	<0.01		0.013	<0.01		<0.01	<0.01	
Maturity			<0.01			<0.01			<0.01	
Addit*Matur			0.071			0.178			0.113	

NS- Not significant, P>0.05; *P<0.05; L- Linear, Q- Quadratic, C- Cubic;

[a,b,c]Means in the same column with different superscripts differed; Addit=Additive; Matur= Maturity.

Similar results were observed in the ADF concentration, where none of the applied additives solubilized the 6-wk guineagrass hays, nevertheless A-20 produced a cubic effect ($P<0.05$) as application rate increased. Nevertheless, for the 8-wk regrowth X-20, Fib and A-20 improved ($P<0.01$) the hydrolysis of the ADF fraction, and this effect was similarly reflected across maturities, where both enzymes decreased ($P<0.01$) the ADF concentration.

Enzyme treatment did not affect the CP concentration of 6-wk and 8-wk hays; however, NH_3 treatment produced greater ($P<0.01$) values at both maturities than those of control and enzyme-treated hays, which agrees with results obtained by Dean *et al.* (2005), who observed that CP concentration of bermudagrass (*Cynodon dactylon*) and bahiagrass (*Paspalum notatum*) hays were increased by ammonia treatment but they were unaffected by enzyme treatment. Brown and Adjei (1995) applied a urea solution

(0, 4, 6, or 8% of the forage DM) to guineagrass hay harvested at different moisture concentrations (25 or 40%) and also observed that CP concentration increased linearly ($P < 0.01$), whereas concentrations of hemicellulose ($P < 0.01$) decreased linearly with increasing amount of urea applied. The same researchers treated guineagrass hays with urea at 0, 4, or 6% of the forage DM. The urea solution was sprayed onto the flat sides of the bales, or applied by low pressure (10 psi) injection. The greatest improvements in CP and NDF concentration and IVOMD were obtained using the low pressure injection method. According to Weiss and Underwood (1995), ammoniation increases CP in the treated forages by retaining about 50 to 80% of the N in the NH_3 applied to the forage, and some of the retained nitrogen is converted by microbes present on the forage into microbial protein whereas the rest is bound in an unknown manner to the forage fiber components.

Effect of fibrolytic enzymes and ammonia on in vitro digestibility

The IVDMD at 6-wk was lower than that reported by Fukushima and Dehority (2000) in two varieties of guineagrass at the late boot stage but higher than that at the seed stage. The IVDMD was lower in the X-20 and A-20 6-wk regrowth-treated hays compared to control treatment. In the 8-wk regrowth hays, only NH_3 treatment increased the IVDMD. The effectiveness of NH_3 at increasing the digestibility of the hays concurs with those of Dean (2005) who observed that only X-20 and ammoniation were effective at improving the 48-h IVDMD of both bermudagrass and bahiagrass silages, and also agrees with that of Zorrilla-Rios *et al.* (1991), who observed that ammoniation increased the IVDMD of wheat (*Triticum aestivum*) straw by approximately 54%. These results also concur with the reports by Brown and Adjei (1995) who observed that ammoniation increased linearly ($P < 0.01$) the *in vitro* OM disappearance (IVOMD) of guineagrass hay as the level of urea application increased. This finding contradicts the conclusion that fibrolytic enzyme application can increase the rate of digestion of forages (Wang and McAllister, 2002), but not the extent of digestion.

The IVNDFD and IVADFD at both maturities were lower than those reported by Ngo and Wiktorsson (2003) in hays of two varieties of guineagrass harvested at 4, 6 and 8-wk regrowths. The IVNDFD of the 6-wk hays was lowered by X-20, Fib and A-20 treatments compared to control treatment, and at 8-wk the NH_3 was the only effective treatment for improving the IVNDFD. Across maturities hays treated with X-20 and Fib showed lower ($P < 0.05$) IVNDFD than control, and the highest values for this variable was obtained in the NH_3-treated hays.

The IVADFD of the 6-wk hays was reduced by X-20, Fib and A-20, but in the 8-wk hays NH_3 and Pr increased the IVADFD. This result indicates that only NH_3 treatment was effective at improving the DM and fiber digestibility of the guineagrass hays and agrees with those obtained by Brown and Adjei (1995) who observed that apparent NDF and ADF digestibilities increased linearly ($P < 0.05$) with increasing levels of urea addition to tropical grass hays.

CONCLUSIONS

This study shows that in the 8-wk regrowth hay the enzymes X-20 and Fibrozyme decreased the NDF and ADF concentration compared to control hay, but only ammoniation increased CP concentration of 6 and 8-wk hays. Ammoniation was the only effective treatment to improve the DM, NDF and ADF digestibility of the 8-wk regrowth hay.

REFERENCES

Aganga, A.A. and Tshwenyane, S. 2004. Potential of Guineagrass (*Panicum maximum*) as forage crop in livestock production. *Pakistan Journal of Nutrition,* 3: 1-4

Bailey, M.J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology,* 23: 257-270.

Bamikole, M.A., Akinsoyinu, A.O., Ezenwa, I., Babayemi, O.J., Akinlade, J. and Adewumi, M.K. 2004. Effect of six-weekly harvests on the yield, chemical composition and dry matter degradability of *Panicum maximum* and *Stylosanthes hamata* in Nigeria. *Grass and Forage Science*, 59: 357-363.

Barrios-Urdaneta, A. and Ventura, M. 2002. Use of dry ammoniation to improve the nutritive value of *Brachiaria humidicola* hay. *Livestock Research for Rural Development,* 14: 56-62.

Bates, D.B., Kunkle, W.E., Chambliss, C.G. and Cromwell, R.P. 1989. Effect of wilting and ammonia treatment of bermudagrass and rhizoma peanut prior to ensiling as round bales. *Florida Beef Cattle Research Report*, University of Florida, IFAS, Gainesville, FL, USA. pp. 14-18.

Brown, W.F. 1993. Cane molasses and cottonseed meal supplementation of ammoniated tropical grass hay for yearling cattle. *Journal of Animal Science,* 71: 3451-3457.

Brown, W.F. and Adjei, M.B. 1995. Urea ammoniation effects on the feeding value of guineagrass (*Panicum maximum*) hay. *Journal of Animal Science,* 73: 3085-3093.

Brown, W.F. and Pate, F.M. 1997. Cottonseed meal or feather meal supplementation of ammoniated tropical grass hay for yearling cattle. *Journal of Animal Science,* 75: 1666-1673.

Christensen, D. 1997. Enzymes: *A New Approach to Enhancing Ruminant Rations*. Feed News. Feed Resource Centre. Volume 2, Issue 2, Saskatoon, Saskatchewan, Canada, pp. 4-5.

Colombatto, D., Mould, F.L., Bhat, M.K., Morgavi, D.P., Beauchemin, K.A. and Owen, E. 2003. Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal micro-organisms *in vitro*. *Journal of Animal Science,* 81: 1040-1050.

Dean, D.B. 2005. *Effect of Fibrolytic Enzymes on the Nutritive Value of Tropical Grasses and the Performance of Dairy Cows*. PhD Thesis. Department of Animal Sciences, University of Florida, Gainesville, FL. USA, 149 pp.

Fukushima, R.S. and Dehority, B.A. 2000. Feasibility of using lignin isolated from forages by solubilization in acetyl bromide as a standard for lignin analyses. *Journal of Animal Science,* 78: 135-3143.

Hristov, A.N., McAllister, T.A. and Cheng, K.J. 1998. Effect of dietary or abomasal supplementation of exogenous polysaccharide-degrading enzymes on rumen fermentation and nutrient digestibility. *Journal of Animal Science,* 76: 3146-3156.

Jung, H.G. and Allen, M.S. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. *Journal of Animal Science,* 73: 2774-2790.

Lewis, G.E., Sanchez, W.K., Hunt, C.W., Guy, M.A., Pritchard, G.T., Swanson, B.I. and Treacher, R.J. 1999. Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. *Journal of Dairy Science* 82: 611-617.

Ministry of Agriculture, Fisheries and Food. 1986. *The Analysis of Agricultural Materials*. Reference Book 427. HMSO, London.

Ngo, V.M. and Wiktorsson, H. 2003. Forage yield, nutritive value, feed intake and digestibility of three grass species as affected by harvest frequency. *Tropical Grasslands,* 37: 101-110.

Noel, R.J. and Hambleton, L.G. 1976. Collaborative study of a semi automated method for determination of crude protein in animal feeds. *Journal of the Association of Official Agricultural Chemists,* 59: 134-140.

Pinos-Rodríguez, J.M., González, S.S., Mendoza, G.D., Bárcena, R., Cobos, M.A., Hernández, A. and Ortega, M.E. 2002. Effect of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. *Journal of Animal Science,* 80: 3016-3020.

Rode, L.M. and Beauchemin, K.A. 1998. *Enzymes to Enhance Utilization of Feed in Dairy Cows*. Research Center, Agriculture and Agri-Food Canada, Box 3000, Lethbridge, AB T1J 4B1, Canada. Available: http://www.wcds.afns.ualberta.ca/Proceedings/1998/ch15.htm Accessed: 2/10/03.

Rode, L.M., Yang, W.Z. and Beauchemin, K.A. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. *Journal of Dairy Science,* 82: 2121-2126.

Vagnoni, D.B., Craig, W.M., Gates, R.N. and Wyatt, W.E. and Southern, L.L. 1995. Monensin and ammoniation or urea supplementation of bermudagrass hay diets for steers. *Journal of Animal Science,* 73: 1793-1802.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3568-3597.

Wang, Y. and McAllister, T.A. 2002. Rumen microbes, enzymes and feed digestion- a review. *Asian-Australian Journal of Animal Sciences,* 15: 1659-1676.

Weiss, B. and Underwood, J. 1995. *Improving Lower Quality Dry Forages by Ammoniation. Ohio State University,* Department of Horticulture and Crop Science. Available: http://ohioline.osu.edu/agf-fact/0015.html Accessed: 12/04/03.

Wood, T.M. and Bhat, M.K. 1988. Methods for measuring cellulase activities. In: *Methods in Enzymology* (Eds. W.A. Wood and S.T. Kellogg), Academic Press, London, UK, 160: 87-112.

Woolford, M.K. and Tetlow, R.M. 1984. The effect of anhydrous ammonia and moisture content on the preservation and chemical composition of rhizoma ryegrass hay. *Animal Feed Science and Technology,* 1: 159-165.

Zorrilla-Rios, J., Horn, G.W., Phillip, W.A. and McNew, R.W. 1991. Energy and protein supplementation of ammoniated wheat straw diets for growing steers. *Journal of Animal Science,* 69: 1809-1819.

Animal Nutrition and Feed Technology (2013) 13: 527-536

Influence of Exogenous Enzymes on *In Vitro* Gas Production Kinetics and Dry Matter Degradability of a High Concentrate Diet[#]

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ABSTRACT

López, D., Elghandour, M.M.Y., Salem, A.Z.M., Vázquez-Armijo, J.F., Salazar, M.C. and Gado, H.M. 2013. Influence of exogenous enzymes on in vitro gas production kinetics and dry matter degradability of a high concentrate diet. Animal Nutrition and Feed Technology, 13: 527-536.

This study was conducted to evaluate the influence of an exogenous enzyme mixture on *in vitro* gas production (GP), *in vitro* dry matter degradability (DMD), metabolizable energy (ME) and short chain fatty acid (SCFA) production in growing lambs fed a high concentrate diet. ZADO® (ENZ) is a powdered, commercially available multi-enzyme feed additive produced from *Ruminococcus flavefaciens*. Four levels of ENZ (*i.e.*, 0, 5, 10 and 20 mg/g DM; or EO, E5, E10 and E20, respectively) were applied directly to the substrate inside the incubation bottles before addition of buffer medium and rumen fluid, and the treatments were assayed in triplicate runs. Addition of ENZ linearly increased ($P<0.05$) GP at 6 and 96 h of incubation and tended ($P=0.08$) to linearly increase GP at 12, 48 and 72 h of incubation. Asymptotic GP was increased linearly ($P=0.05$) as the level of ENZ increased and the lag time decreased linearly ($P=0.003$). Concurrently, DMD increased linearly ($P<0.001$) as the level of ENZ increased, but level of ENZ had no effect on SCFA and ME. Finally, level of ENZ had no influence on rate of gas production. Results suggest that this enzyme preparation has potential to improve efficiency of utilization of high concentrate diets fed to growing lambs.

Key words: Exogenous enzymes, *In vitro* gas production, Degradability, Lambs.

INTRODUCTION

Use of enzymes as feed additives in ruminant diets has attracted considerable interest in recent years. However, there is increasing evidence indicating that the mode

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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of action of exogenous enzymes in ruminants combines pre- and post-feeding effects (Colombatto *et al.*, 2007). Of these pre-feeding effects, enzyme application rate and available enzyme/feed interaction time appear as important (Yang *et al.*, 2000). Responses to enzyme application level have been somewhat variable, with quadratic responses most commonly observed (Beauchemin *et al.*, 2003) in which maximum benefits occur at intermediate enzyme addition levels. Other reports have emphasized the importance of the enzyme/feed interaction time, since addition of enzyme in animal feeds may create a stable enzyme/feed complex which protects free enzymes from proteolysis in the rumen (Kung *et al.*, 2000).

Improvements in ruminant production with supplemental fibrolytic enzymes are generally attributed to increased ruminal fiber digestion, but the mechanism by which this increase occurs are not completely understood. Numerous potential mechanisms have been proposed (Beauchemin *et al.*, 2003), including pre-ingestive and ruminal effects such as direct hydrolysis (Moharrery *et al.*, 2009), structural changes in the fiber, increased ruminal microbial attachment (Colombatto *et al.*, 2003), stimulation of ruminal microbial populations and synergism with ruminal microbial enzymes.

As published opinions of scientists vary, no firm conclusions can be drawn about the effects of exogenous enzymes in animal nutrition. However, effects of exogenous enzymes on *in vitro* fermentation kinetics depend on the source of enzyme (Salem *et al.*, 2012), level and activity of the enzyme (Jalilvand *et al.*, 2008) and the physical chemistry properties of substrate (Kung *et al.*, 2000; Moharrery *et al.*, 2009). Synergistic effects between exogenous enzymes and ruminal micro-organisms and enzymes (Morgavi *et al.*, 2000), and/or the ruminal environment (Colombatto *et al.*, 2003) have also been reported.

Recent research has shown that xylanase-based products used as an additive to non-lactating dairy cow diets had no effects on potential dry matter (DM) and acid detergent fiber (ADF) disappearance (Phakachoed *et al.*, 2012). Pre-treatment of forage with fibrolytic enzymes can solubilize some fiber and improve digestibility at short incubation times (Moharrery *et al.*, 2009). It appears that effective enzymes work best by removing structural barriers which retard microbial colonization of digestible fractions (Colombatto *et al.*, 2003) to increase rate of degradation. Exogenous fibrolyitc enzymes also seem to work better at close to neutral ruminal pH (Colombatto *et al.*, 2007).

However, there are some inconsistencies on effects of exogenous enzyme levels on ruminal fermentation kinetics. Some research has shown that efficiencies of forage utilization were increased at increasing levels of exogenous enzymes (Miller *et al.*, 2008) whereas others suggest that exogenous enzymes produced better results at a particular level, rather than showing a dose response (Jalilvand *et al.*, 2008). The objective of the present research was to evaluate the dose response of exogenous enzyme addition on *in vitro* gas production kinetics and energy utilization of growing lambs fed a high concentrate diet.

MATERIALS AND METHODS

Substrates and enzyme product

Three individual samples of a high concentrate lamb diet (HCD, Table 1) were randomly collected in triplicate from different fields. Samples were dried at 70°C for 48 h in a forced air oven to constant weight, ground in a hammer mill to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical components and *in vitro* GP mixed with four doses of ZADO® enzyme preparation mixture (ENZ), a patented (Patent No.: 22155, Cairo, Egypt) commercially available multi-enzyme feed additive in a powder form produced from *Ruminococcus flavefaciens* and manufactured by the Academy of Scientific Research and Technology in Cairo (Egypt). Prior to our research, the enzyme mixture was assayed for several enzymatic activities and found to contain (per g ENZ) 7.1 units of endoglucanase, 2.3 units of xylanase, 61.5 units of α-amylase and 29.2 units of protease activity. Doses of ENZ inclusion were (per g DM of HCD): control (0 mg, no enzyme - E0), low (5 mg - E5), medium (10 mg - E10) and high (20 mg - E20).

In vitro incubation

Rumen fluid was collected by stomach tube from 4 growing lambs (Katahdin×Pelibuey, live weight 24±0.3 kg) fed HCD *ad libitum* (Table 1). Samples (1 g) of substrate (*i.e.,* HCD) were weighed into 120 ml serum bottles. Enzyme (ENZ) doses (*i.e.*, 0, 5, 10, 20 mg/g DM) were added directly on to the substrate immediately before addition of buffer medium and rumen fluid.

Table 1. Ingredient and chemical composition of the growing lamb's diet in g/kg DM (adapted from Salem *et al.*, 2012)

Ingredient composition	
Soybean meal	220
Alfalfa hay	150
Sorghum grain	550
Fishmeal	35
Mineral/vitamin premix[1]	25
Salt	20
Chemical composition	
Organic matter	911
Crude protein	219
Ether extract	119
Neutral detergent fiber	141
Acid detergent fiber	59
Lignin	21

[1]Mineral/vitamin premix (25) (Vitamin A (12 000 000 IU), Vitamin D3 (2 500 000 IU), Vitamin E (15 000 IU), Vitamin K (2.0 g), Vitamin B1 (2.25 g), Vitamin B2 (7.5 g), Vitamin B6 (3.5 g), Vitamin B12 (20 mg), Pantotenic acid (12.5 g), Folic acid (1.5 g), Biotin (125 mg), Niacin (45 g), Fe (50 g), Zn (50 g), Mn (110 g), Cu (12 g), I (0.30 g), Se (200 mg), Co (0.20 g).

Ruminal contents were obtained from each sheep immediately before morning feeding, mixed and strained through 4 layers of cheesecloth into a flask with an O_2-free headspace. Ten ml of particle-free ruminal fluid was then added to each serum bottle and 40 ml of buffer solution (Goering and Van Soest, 1970), with no trypticase immediately added in a proportion 1: 4 (v/v).

A total of 108 bottles, 3 bottles for each ENZ dose (*i.e.*, 0, 5, 10, 20 ml/g DM) in triplicate samples of HCD in 3 runs for different weeks plus 3 bottles as blanks (*i.e.,* rumen fluid only), were incubated for 96 h. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in the incubator at 39°C. The volume of gas produced was recorded at incubation times of 2, 4, 6, 8, 10, 12, 24,48, 72 and 96 h after inoculation using the Reading Pressure Technique (RPT; DELTA OHM, Italy) of Mauricio *et al*. (1999). At the end of the incubation at 96 h, bottles were uncapped and the pH was measured immediately using a pH meter (GLP 22, Crison Instruments, Barcelona, Spain). The fermentation was then stopped by swirling the bottles in ice.

Dry matter degradability

At the end of incubation (*i.e.* 96 h), contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100 to 160 μm, Pyrex, Stone, UK). Fermentation residues were dried at 105°C overnight to estimate potential DM disappearance. Loss in weight after drying was used as the measure of undegradable DM. The DM degradability at 96 h of incubation (*i.e.,* dry matter degradability; mg/g DM) was calculated as the difference between DM content of substrate and its undegradable DM.

Chemical analyses and assay of enzymatic activity

Samples of HCD were analysed for DM (934.01), ash (942.05), N (954.01) and EE (920.39) according to AOAC (1997). Neutral detergent fiber (NDF; Van Soest and Mason, 1991), acid detergent fiber (ADF) and lignin (AOAC, 1997; 973.18) analyses were carried out using an ANKOM 200 Fiber Analyzer Unit (ANKOM Corporation, Macedon, NY, USA). The NDF was assayed without use of an alpha amylase but with sodium sulfite in the NDF. Both NDF and ADF are expressed without residual ash.

Endoglucanase activity of ZADO® was assayed by liberating glucose from carboxymethyl cellulose, which was determined calorimetrically using alkaline copper reagent as described by Robyt and Whelan (1972). One unit of endoglucanase catalyzes liberation of one mmol of glucose/min from sodium carboxymethyl cellulose at 40°C and pH 4.5. The α-amylase was assayed by its ability to produce reducing groups from starch, which were measured by reduction of 3,5-dinitrosalicylic acid (Bernfeld, 1955). One unit of α-amylase catalyzes liberation of one mmol of reducing groups/min from soluble starch at 25°C and pH 6.0, calculated as maltose equivalents. Protease activity was determined by hydrolysis of dimethyl casein (DMC) and the liberated amino acids

were determined using 2,4,6-trinitrobenzene sulfonic acid (Lin *et al.*, 1969). One DMC unit catalyzes cleavage of one mmol of peptide bond/min from DMC at 25°C and pH 7.0 expressed in terms of newly formed terminal amino groups. Xylanase catalyzes hydrolysis of xylan from oat spelt, and the reducing groups liberated were determined using alkaline copper reagent (Robyt and Whelan, 1972). One unit catalyzes the liberation of one mmol reducing groups/h from xylan at 37°C and pH 5.5, expressed as xylose equivalents.

Calculations

To estimate kinetic parameters of GP results (ml/g DM) were fitted using the NLIN option of SAS (2002) according to the France *et al.* (2000) using the model:

$$GP = b \times (1 - e^{-c^{(t-L)}})$$

Where, GP is the volume of GP at time t; b is the asymptotic GP (ml/g DM); c is the rate of GP (/h), and L (h) is the discrete lag time prior to GP.

Metabolizable energy (ME, MJ/kg DM) was estimated according to Menke *et al.* (1979) as:

$$ME = 2.20 + 0.136\ GP\ (ml/0.5g\ DM) + 0.057\ CP\ (g/kg\ DM)$$

Where, GP is net GP in ml from 200 mg of dry sample after 24 h of incubation.

Short chain fatty acids concentration (SCFA) was calculated according to Getachew *et al.* (2002) as:

$$SCFA\ (mmol/200\ mg\ DM) = 0.0222\ GP - 0.00425$$

Where, GP is the 24 h net gas production (ml/200 mg DM).

Statistical analyses

Data of *in vitro* ruminal GP and fermentation parameters were analysed as a randomized design using the PROC MIXED procedure of SAS (2002). Data of each of the 3 runs within the same sample of HCD were averaged prior to statistical analysis. Mean values of each individual sample within each HCD sample (3 samples of each) were used as the experimental unit. The statistical model was:

$$Y_{ij} = \mu + Z_j + \varepsilon_{ij}$$

Where, Y_{ijk} represents every observation of the i^{th} lamb diet when incubated in the j^{th} ENZ doses, Z_j is the ENZ doses and ε_{ijk} is the experimental error. Tukey's test was used for the multiple comparisons among mean values for each HCD sample and linear and quadratic effects were calculated at $P < 0.05$.

RESULTS

Cumulative *in vitro* GP (ml/g DM) from the HCD treated with different levels of ENZ and incubated in rumen liquor of growing lambs is shown in Table 2. In general,

lower levels of ENZ application tended to be more effective in increasing fermentation kinetics of HCD of growing lambs in the early stages of incubation, whereas higher levels of ENZ were more effective at the mid- and late stages of incubation. Specifically, ENZ linearly increased ($P<0.05$) GP at 6 and 96 h of incubation and tended ($P=0.08$) to linearly increase GP at 12, 48 and 72 h of incubation without quadratic effects.

Table 2. Cumulative *in vitro* gas production (ml/g DM) from a high concentrate diet treated with different levels of enzyme (ENZ) and incubated in rumen liquor from growing lambs

Time (h)	ENZ level†				SEM	P Linear
	E0	E5	E10	E20		
6	74.5	81.1	83.7	87.5	2.22	0.006
12	162.4	196.8	168.5	174.7	3.34	0.048
24	259.1	265.3	263.0	271.1	4.45	0.122
48	320.1	323.5	323.9	332.4	4.73	0.076
72	331.9	334.1	336.0	344.4	5.82	0.057
96	334.1	336.1	338.5	346.8	6.98	0.052

†ENZ: ZADO® enzyme added at 0 (E0), 5 (E5), 10 (E10) and 20 (E20) mg/g DM, respectively.

The GP parameters (b, c and L phase), *in vitro* DM degradability (DMD), metabolizable energy (ME) and short chain fatty acids (SCFA) of the HCD fed to growing lambs with different levels of ENZ is in Table 3. The asymptotic GP increased linearly ($P<0.05$) as the level of ENZ increased and the lag time decreased linearly ($P=0.003$) as the level of ENZ increased. Concurrently, DMD increased linearly ($P<0.001$) as level of ENZ increased, but level of ENZ had no effect on SCFA and ME. Additionally, level of ENZ had no influence on the rate of GP. Generally, there were not any quadratic effects on the fermentation parameters.

Table 3. Gas production parameters†, *in vitro* dry matter degradability (DMD, %), metabolizable energy (ME, MJ/kg DM) and short chain fatty acids (SCFA, mmol) of the high concentrate diet fed to growing lambs with different levels enzyme (ENZ)

	ENZ levels‡				SEM	P Linear
	E0	E5	E10	E20		
Asymptotic, ml/g DM	334.7	336.5	339.1	347.3	5.83	0.050
Rate of gas,/h	0.069	0.071	0.067	0.068	0.0051	0.406
Lag time, h	2.34	2.12	1.78	1.73	0.114	0.003
DMD	70.9	80.9	82.0	84.3	0.96	<.001
ME	10.2	10.4	10.3	10.7	1.25	0.123
SCFA	1.15	1.17	1.16	1.20	0.135	0.122

†Parameters of gas productions were estimated according to the model of France *et al.* (2000).

‡ENZ, ZADO® enzyme added at 0 (E0), 5 (E5), 10 (E10) and 20 (E20) mg/g DM, respectively.

DISCUSSION

The substrate incubated with ENZ was similar to the diet fed to growing lambs to evaluate effects of ENZ on GP kinetics of similar diets to reflect the properties of the reaction of ENZ to those feeds. The GP and DMD appeared to be related to the level of ENZ applied (Tables 2 and 3), which is consistent with numerous recent studies which have shown that adding exogenous fibrolytic enzymes to ruminant diets improved digestion of DM and fiber *in vitro* (Moharrery *et al.*, 2009) and *in vivo* (Salem *et al.*, 2012). Eun *et al.* (2007) reported that *in vitro* GP and the DM degradation rate were useful in identifying changes in substrate availability due to enzyme addition.

Effects of ENZ levels on GP

Gas production *in vitro* appears related to the chemical composition of the feed, in particular to the fiber content and its structural polysaccharides (Jalilvand,*et al.*, 2008). As the results of our study suggest, the particular ENZ used here has potential as an influence to GP kinetics. Cumulative GP after 6 h of incubation increased ($P<0.01$) with ENZ addition compared to E0 but, as level of ENZ had no effect on GP at other incubation times, this suggests that the *Ruminococcus flavefaciens* in the ENZ might have caused stimulation of *in vitro* ruminal fermentation at early incubation times, but not at mid times. The initial fermentation of high concentrate diets, in ruminal fluid is generally enzyme-limited. However, addition of ENZ at 72 h ($P=0.057$) and 96 h ($P=0.052$) increased GP during the later period of fermentation, which may be a reflection of an increase in bacterial numbers, and hence, hydrolytic capacity of the ruminal fluid. This view is similar to previous hypotheses that exogenous ENZ increased fibrolytic activity due to increased numbers of ruminal microbes (Colombatto *et al.*, 2003), and increased bacterial attachment and synergistic effects with hydrolysis of ruminal micro-organisms. Another report showed that a fibrolytic enzyme preparation increased numbers of cellobiose-utilizing, xylanolytic and amylolytic bacteria, but had no effect on numbers of cellulolytic bacteria (Nsereko *et al.*, 2002) and that the population density of *Ruminobacter amylophilus* was increased by the high enzyme treatment, while *Selenomonas ruminantium* tended to increase linearly with increasing levels of enzyme (Chung *et al.*, 2012).

That the asymptotic GP was increased linearly as the level of ENZ increased indicates that the higher level of ENZ improved rumen fermentation kinetics. However, these results contrast with Jalilvand *et al.* (2008), who found that effects of addition of ENZ to different forages on GP kinetics were negligible, which suggests that quality of the diet is important, and that effects of ENZ depend on the fiber content, structural polysaccharide composition of the substrate and the differences in enzyme composition.

Enzymes produced by a variety of microbes are capable of degrading lignocellulosic materials to SCFA, but require substantive rumen retention time (Kumar *et al.*, 2009). In our study, the lag time decreased linearly as the level of ENZ increased, indicating that ENZ could degrade complex substrates to simpler ones at early stages of fermentation to allow faster ruminal microbial colonization and

fermentation (Colombatto *et al.*, 2003). Adding the ENZ at more than 2.55 l/g of DM did not increase rate of GP further, suggesting that the maximal level of stimulation was already achieved at lower ENZ concentrations. Enzyme addition tended to affect asymptotic GP, but had no effect on rate of GP, which is consistent with previous studies using other types of enzymes (Jalilvand *et al.*, 2008).

Effect of ENZ levels on DMD, ME and SCFA

The ENZ linearly increased ($P<0.001$) DMD) of this HCD, which is consistent with Miller *et al.* (2008) but contrasts with Jalilvand *et al.* (2008) who reported a quadratic effect with increasing level of ENZ. This ENZ effect may be due to the efficiency of ENZ addition which could degrade the complex lignocelluloses substrate of HCD to simpler compounds which might change the surface structure of substrates or destroy the chemical bond of lignocelluloses, making them more accessible to ruminal microbial degradation and/or promote colonization of ruminal microbes and efficiency of fermentation. This linear increase of DMD with increasing levels of ENZ is consistent with previous studies using the same enzyme mixture (Gado *et al.*, 2011; Gado *et al.*, 2009; Salem *et al.*, 2013) and other exogenous enzymes (Mattéotti *et al.*, 2012).Recent work has demonstrated that supplementing diets of ruminants with exogenous enzymes can improve DM intake, feed utilization and animal performance by enhancing DM degradation *in situ* (Krueger and Adesogan, 2008), *in vitro* (Colombatto *et al.*, 2007; Moharrery *et al.*, 2009) and *in vivo* (Salem *et al.*, 2012, 2013).

While the mechanism by which our ENZ improved the digestion of forage is still poorly understood, several modes of action are suggested. These include that ENZ increased ruminal microbial colonization on the surface of feed particles (Yang *et al.*, 2000) to enhance attachment and improve access to the matrix surface by ruminal microbes to accelerate rate of digestion (Jalilvand *et al.*, 2008), or that ENZ enhanced the hydrolytic ability of ruminal microbes due to enzyme activities or increased synergism with rumen microbial enzymes (Morgavi *et al.*, 2000). Unfortunately, we did not measure enzyme activities in this particular study, which does not allow to draw a final conclusion.

That the ENZ had no impact on ME and SCFA suggests that energy was not the limiting nutrient in our HCD. Beauchemin *et al.* (2003) showed that ENZ increased digestible energy intake of animal when the substrate was fiber-rich material and energy was the limiting nutrient in the diet. The lack of ENZ effect on SCFA suggests that exogenous enzymes did not change the diversity of the ruminal microbial communities enough to affect SCFA, which contrasts with Gado *et al.* (2009) and Salem *et al.* (2013) who reported that supplementation of enzymes increased total SCFA concentrations.

Results suggest that our exogenous enzyme mixture produced from *Ruminococcus flavefaciens* holds potential to improve efficiency of utilization of high concentrate diets fed to growing lambs, as evidenced by increased gas production, *in vitro* dry matter degradability and reduced lag time. *In vivo* studies are required to confirm these findings.

Addition of exogenous enzymes to ruminant diets is one of the effective additives in animal nutrition. Enzymes have a potential impacts on improving the nutritive utilization of high concentrate diet fed to growing animals due improving the ruminal activities. Research is needed to understand the mode of action of this enzyme product which could play an important role in future ruminant production system.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from the IAEA, Vienna, Austria, Research Contract number MEX16307 within the D3.10.27 Coordinated Research Project.

REFERENCES

AOAC. 1997. *Animal Feeds: Official Methods of Analysis of AOAC International,* 16th ed., Vol. 1. AOAC International, Virginia, VA, USA.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science,* 81: E37-E47.

Bernfeld, P. 1955. Amylases: α and β. In: *Methods in Enzymology* (Eds. S.P. Colowick and N.O. Kaplan), Academic Press, New York, pp. 149-158.

Chung, Y.H., Zhou, M., Holtshausen, L., Alexander, T.W., McAllister, T.A., Guan, L.L., Oba, M. and Beauchemin, K.A. 2012. A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions. *Journal of Dairy Science,* 95: 1419-1427.

Colombatto, D., Morgavi, D.P., Furtado, A.F. and Beauchemin, K.A. 2003. Screening of exogenous enzymes for ruminant diets: Relationship between biochemical characteristics and *in vitro* ruminal degradation. *Journal Animal Science*, 81: 2628-2638.

Colombatto, D., Mould, F.L., Bhat, M.K. and Owen, E. 2007. Influence of exogenous fibrolytic enzyme level and incubation pH on the *in vitro* ruminal fermentation of alfalfa stems. *Animal Feed Science and Technology,* 137: 150-162.

Eun, J.S., Beauchemin, K.A. and Schulze, H. 2007. Use of an *in vitro* fermentation bioassay to evaluate improvements in degradation of alfalfa hay due to exogenous feed enzymes. *Animal Feed Science and Technology,* 135: 315-328.

France, J., Dijkstra, J., Dhanoa, M.S., Lopez, S. and Bannink, A. 2000. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed *in vitro*: derivation of models and other mathematical considerations. *British Journal Nutrition*, 83: 143-150.

Gado, H.M., Salem, A.Z.M., Odongo, N.E. and Borhami, B.E. 2011. Influence of exogenous enzymes ensiled with orange pulp on digestion and growth performance in lambs. *Animal Feed Science and Technology,* 165: 131-136.

Gado, H.M., Salem, A.Z.M., Robinson, P.H. and Hassan, M. 2009. Influence of exogenous enzymes on nutrient digestibility, extent of ruminal fermentation as well as milk production and composition in dairy cows. *Animal Feed Science and Technology,* 154: 36-46.

Getachew, G., Makkar, H.P.S. and Becker, K. 2002. Tropical browses: contents of phenolic compounds, *in vitro* gas production and stoichiometric relationship between short chain fatty acid and *in vitro* gas production. *Journal of Agriculture Science*, 139: 341-352.

Goering, M.K. and Van Soest, P.J. 1970. Forage fiber analysis (apparatus, reagents, procedures and some applications). Agriculture Handbook, No 379. Agricultural Research Service, USDA. Washington, DC, USA.

Jalilvand, G., Odongo, N.E., López, S., Naserian, A., Valizadeh, R., Eftekhar Shahrodi, F., Kebreab, E. and France, J. 2008. Effects of different levels of an enzyme mixture on *in vitro* gas production parameters of contrasting forages. *Animal Feed Science and Technology,* 146: 289-301.

Krueger, N.A. and Adesogan, A.T. 2008. Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. *Animal Feed Science and Technology,* 145: 84-94.

Kumar, P., Barrett, D.M., Delwiche, M.J. and Stroeve, P. 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Industrial and Engineering Chemistry Research*, 48: 3713-3729.

Kung, L., Treacher, R.J., Nauman, G.A., Smagala, A.M., Endres, K.M. and Cohen, M.A. 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. *Journal of Dairy Science,* 83: 115-122.

Lin, Y., Means, G.E. and Feeney, R.E., 1969. An assay for carboxypeptidases A and B on polypeptides from protein. *Analytical Biochemistry*, 32: 436-445.

Mattéotti, C., Bauwens, J., Brasseur, C., Tarayre, C., Thonart, P., Destain, J., Francis, F., Haubruge, E., De Pauw, E., Portetelle, D. and Vandenbol, M. 2012. Identification and characterization of a new xylanase from gram-positive bacteria isolated from termite gut (*Reticulitermes santonensis*). *Protein Expression and Purification*, 83: 117-127.

Miller, D.R., Elliott, R. and Norton, B.W. 2008. Effects of an exogenous enzyme, Roxazyme® G2 Liquid, on digestion and utilisation of barley and sorghum grain-based diets by ewe lambs. *Animal Feed Science and Technology,* 140: 90-109.

Moharrery, A., Hvelplund, T. and Weisbjerg, M.R. 2009. Effect of forage type, harvesting time and exogenous enzyme application on degradation characteristics measured using *in vitro* technique. *Animal Feed Science and Technology,* 153: 178-192.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., Iwaasa, A.D., Yang, W.Z., McAllister, T.A. and Wang, Y. 2000. Synergy between ruminal fibrolytic enzymes and enzymes from *Trichoderma longibrachiatum*. *Journal of Dairy Science,* 83: 1310-1321.

Nsereko, V.L., Beauchemin, K.A., Morgavi, D.P., Rode, L.M., Furtado, A.F., McAllister, T.A., Iwaasa, A.D., Yang, W.Z. and Wang, Y. 2002. Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. *Canadian Journal of Microbiology*, 48: 14-20.

Phakachoed, N., Lounglawan, P. and Suksombat, W. 2012. Effects of xylanase supplementation on ruminal digestibility in fistulated non-lactating dairy cows fed rice straw. *Livestock Science*, 149: 104-108.

Robyt, J.F. and Whelan, W.J. 1972. Reducing value methods for maltodextrins: I. Chain-length dependence of alkaline 3,5-dinitrosalicylate and chain-length independence of alkaline copper. *Analytical Biochemistry*, 45: 510-516.

Salem, A.Z.M., Gado, H.M., Colombatto, D.B. and Eghandour, M.M.Y. 2013. Effect of exogenous enzymes on nutrient digestibility, ruminal fermentation and growth performance in beef steers. *Livestock Science*, 154: 69–73.

Salem, A.Z.M., Hassan, A.A., Gado, H.M., Khalil, M.S., Simbaya, J. and Alsarsy, H. 2012. Effect of sun-drying and exogenous enzymes on nutrients intake and digestibility as well as nitrogen utilization in sheep fed *Atriplex halimus* foliages. *Animal Feed Science and Technology,* 171: 128-135.

SAS Institute 2002. SAS User's Guide: Statistics. Ver 9.0. SAS Institute. Cary, NC, USA.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B. and France, J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology,* 48: 185-197.

Van Soest, P.J. and Mason, V.C. 1991. The influence of the Maillard reaction upon the nutritive value of fibrous feeds. *Animal Feed Science and Technology,* 32: 45-53.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science,* 83: 2512-2520.

Animal Nutrition and Feed Technology (2013) 13: 537-550

Effect of Fibrolytic Enzyme Application on the Digestibility of Corn Silage, Alfalfa Hay, Concentrates, and Complete Diets under Simulated Ruminal and Preruminal Conditions#

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ABSTRACT

Arriola, K.G. and Adesogan, A.T. 2013. Effect of fibrolytic enzyme application on the digestibility of corn silage, alfalfa hay, two concentrates, and complete diets under simulated ruminal and preruminal conditions. Animal Nutrition and Feed Technology, 13: 537-550.

Previous work showed that when added to the total mixed ration (TMR) prior to feeding, a fibrolytic enzyme containing 733U/ml of endoglucanase activity, 3630 U/ml of xylanase activity, and 0.0002 umol/mg/minute of esterase activity improved dry matter digestibility (DMD) and neutral detergent fiber digestibility (NDFD) and the level and efficiency of milk production by dairy cows. This study determined if the enzyme exerts its hydrolytic effect on different dietary substrates prior to ingestion or within the rumen and determine which dietary components were most affected by enzyme action. Substrates evaluated included corn silage (CS), alfalfa hay (AH), low (LC) and high (HC) - energy concentrates (22 and 37% corn meal) and low- (33%) and high- (48%) concentrate total mixed rations (TMRL and TMRH). Substrates were incubated in a buffer or a buffer-enzyme solution in triplicate for up to 24 h and chemical composition and DM disappearance were measured. In addition, DMD and NDFD were determined after untreated or enzyme-treated substrates were incubated in water (W) or ruminal fluid (RF) for a further 24 h after the initial incubation in the buffer or buffer-enzyme solution. Application of the enzyme reduced concentrations of NDF and hemicellulose, increased water-soluble carbohydrate (WSC) concentration and DM disappearance. Incubation of enzyme-treated substrates in RF resulted in greater DMD than incubation in W except for AH, which had similar DMD in both media. Enzyme addition increased DMD and NDFD in W by 10 and 39% respectively, but had no effect on DMD and NDFD in RF; suggesting that preingestive effects of the enzyme were greater than ruminal effects. Enzyme effects on NDF, WSC, and hemicellulose concentration or DMD and NDFD in W or RF did not depend on the substrate. Therefore, this study provided no evidence that the enzyme preferentially hydrolyzed specific substrates and it suggested that preingestive effects of the enzyme were greater than ruminal effects under the conditions of this study.

Key words: Enzyme, *In vitro*, Corn Silage, Alfalfa.

INTRODUCTION

Exogenous fibrolytic enzyme products usually are applied to the diet before feeding because a preingestive enzyme-feed interaction is necessary for any significant

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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beneficial effects on ruminal digestion to be realized (Lewis *et al.,* 1996; McAllister *et al.,* 1999). The close enzyme-feed association may enable partial hydrolysis of NDF and ADF (Krause *et al.,* 1998) that causes a release of reducing sugars (Hristov *et al.,* 1996; Krueger and Adesogan, 2008). The hydrolysis also may modify plant cell wall structure (Feng *et al.,* 1996) and thereby increase fiber digestion. Another reason for applying enzymes to feed prior to ingestion is to enhance binding of the enzyme to the feed, thereby increasing the resistance of the enzymes to ruminal proteolysis (Fontes *et al.,* 1995).

Despite the evidence for preingestive enzyme action, most attention has focused on ruminal enzyme effects. For instance, Beauchemin *et al.* (2003) stated that most of the improvements in forage quality resulting from exogenous fibrolytic enzyme application are attributable to ruminal effects. Yang *et al.* (1999) reported that most of the effect of exogenous enzymes in diets of lactating dairy cows occurred in the rumen. Yet infusion of exogenous enzymes into the rumen is not considered an effective method of enzyme application (Lewis *et al.,* 1996; Treacher *et al.,* 1997; Hristov *et al.,* 2000; Sutton *et al.,* 2003) because the proper binding of enzymes to feeds that optimizes efficacy occurs in the preingestive phase. Enzyme application to forage several months prior to feeding increased *in vivo* digestibility of DM and NDF in beef steers, whereas enzyme application at feeding had no effect (Krueger *et al.,* 2008), suggesting that an important preingestive effect occurred in the former treatment. Studies directly comparing preingestive versus ruminal enzyme action are needed to quantify the relative importance of these sites of enzyme action and to inform guidelines about the timing of enzyme application to feeds.

Eun and Beauchemin (2007) reported that application of a certain mixture of developmental fibrolytic enzymes (FF and FT, Dyadic International, Jupiter, FL) improved the *in vitro* NDF digestibility of both forages by over 20%. Arriola *et al.* (2011) reported that adding the same enzyme mixture to diets of lactating dairy cows did not affect DMI but tended to increase milk production and therefore increased the efficiency of milk production. These effects were attributed to improved nutrient digestion and ruminal energy utilization. However, the dietary component most affected by the enzyme was not known. Studies aimed at determining the ideal dietary component to which enzymes should be added have not produced consistent results. Milk production by dairy cows has been improved by adding enzymes to the forage (Lewis *et al.,* 1999; Kung *et al.,* 2000) or concentrate (Rode *et al.,* 1999; Yang *et al.,* 2000) portions of diets or to the TMR (Beauchemin *et al.,* 1999) in some studies but not others (Phipps *et al.,* 2000; Sutton *et al.,* 2003; Elwakeel *et al.,* 2007). Most of such studies have not involved concurrent enzyme addition to different dietary fractions therefore little is known about the target dietary fraction to which enzymes should be added.

The objectives of this study were to compare simulated preingestive and ruminal effects of a fibrolytic enzyme on forages, concentrates, and TMR used by Arriola *et al.* (2011) and to determine which dietary components were most affected by enzyme

action. The hypotheses were that the effect of the enzyme in the simulated rumen would exceed the simulated preingestive effect and the enzyme would exert the greatest effect on the forage component of the diet.

MATERIALS AND METHODS

Dietary substrates

Samples of the alfalfa hay (AH), corn silage (CS), low corn (20%; LC) and high corn (34%; HC)- concentrates, and low- (33%) and high- (48%) concentrate TMR (TMRL and TMRH) from the study of Arriola *et al.* (2011) were used as substrates in this study. The substrates were dried in a forced-air oven at 60°C for 48 h, ground to pass through a 1-mm screen using a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA) and stored in air-tight plastic bags.

Enzyme activity

The enzyme mixture was the same developmental enzyme from Dyadic International, Inc. (Jupiter, FL) used by Arriola *et al.* (2011). Xylanase activity (EC 3.2.1.8) measured using the assay of Bailey *et al.* (1992) was 3630 U/ml with oat spelt xylan as the substrate (Sigma Chemical Company, St. Louis, MO, USA). Endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) activities measured with the Wood and Bhat (1988) assays were 733 U/ml and 2 U/ml using 1% (w/v) of carboxymethyl cellulose or 1% (w/v) solution of microcrystalline cellulose (Avicel, Sigmacell 50; Sigma; Chemical Company, St. Louis, MO, USA) as substrates, respectively. One unit of activity of the respective enzymes is defined as micromoles of xylose or glucose released per min. per g. Assay conditions were 39°C and pH 6.0. Release of ferulic acid activity was measured using the assay of Mastihuba *et al.* (2002) and the result was 0.0002 μmol of ferulic acid/min mg^{-1}.

In vitro fermentation and degradability

Four experiments were conducted to test the experimental hypotheses. In Experiment 1, the objective was to determine effects of enzyme addition on chemical composition. Exactly 6.8 mg of the fibrolytic enzyme was diluted in 4 ml of citrate phosphate buffer (pH 6.0) and applied to 2 g of each substrate in beakers in triplicate at 23°C. In addition, beakers containing each substrate and the buffer alone were also prepared and regarded as respective controls. After 8 h, beaker contents were dried overnight at 60°C in a forced-draft oven. Concentrations of NDF and ADF were analysed sequentially using the method of Van Soest *et al.* (1991) in an ANKOM® Fiber Analyzer (ANKOM Technologies, Macedon, NY, USA). Heat stable α-amylase and sodium sulfite were used in the NDF analysis. Hemicellulose was calculated by difference from NDF and ADF. Water-soluble carbohydrate concentration was determined after water extraction, acid hydrolysis, and a colorimetric reaction with potassium ferricyanide (Hall *et al.,* 1999).

In Experiment 2, the objective was to determine effects of enzyme addition on substrate disappearance in a buffer. Exactly 3.4 mg of the fibrolytic enzyme was diluted in 2 ml of citrate phosphate buffer (pH 6.0) and applied to 1 g of each substrate within 250 ml culture bottles in triplicate. After 24 h of incubation at 23°C in the buffer alone or the buffer-enzyme solution, contents of culture bottles were filtered through a Whatman No. 541 filter paper (#09851D, Fisher Scientific, Pittsburgh, PA, USA), and residues were dried at 60°C overnight to determine DM disappearance in the buffer in each of 2 runs.

In Experiment 3 the objective was to compare the DM and NDF digestion of enzyme-treated substrates under simulated preingestive and ruminal conditions. Substrates were incubated for 24 h in the buffered-enzyme solution as in Experiment 2 in each of 3 runs. Subsequently, 40 ml of either distilled water (W) or buffered-ruminal fluid (RF) was added to each culture bottle, and the suspensions were incubated at 39°C for 24 h. Incubation in W was used to simulate preingestive effects of the enzyme, whereas incubation in RF simulated ruminal effects. The RF was collected by aspiration from a non-lactating non-pregnant Holstein cow, filtered through two layers of cheesecloth, immediately transported in a pre-warmed thermos flask to the laboratory, and mixed (1: 2 ratio) under a CO_2 stream with the anaerobic culture medium of Tilley and Terry (1963). Ruminal fluid donor cows were fed bermudagrass hay *ad libitum* supplemented with 750 g of soybean meal daily. The culture medium had been warmed to 39°C to avoid exposing micro-organisms to cold shock. After the 24 h incubation in RF or W, the contents of the culture bottles were filtered through Whatman No. 541 filter paper and residues were dried at 60°C overnight to determine DM digestibility. The DM and NDF concentrations of substrates and digestion residues were measured as described previously and DM (DMD) and NDF digestibility (NDFD) were calculated. Even though substrate disappearance in the buffer or W did not involve enzymic or microbial digestion, DMD and NDFD will be used to describe solubility in these media for simplicity.

In Experiment 4 the objective was to determine effects of enzyme addition on DM and NDF digestibility under simulated preingestive and ruminal conditions. Substrates were prepared and incubated in the buffered enzyme solution or in the buffer alone for 24 h. Subsequently, 40 ml of either W or RF was added to each culture bottle, and the suspensions were incubated at 39°C for 24 h. Dry matter and NDF digestibility were determined as in Experiment 3. A 5 ml aliquot of the RF filtrate was frozen (-20°C) for volatile fatty acid (VFA), pH and NH_3-N analysis. Filtrate samples containing ruminal fluid were analysed for VFA using a Gas Chromatography system (Perkin Elmer Autosystem XL, Waltham, MA) containing a Supelco (Sigma Aldrich, St. Louis, MO) packed column with the following specifications: 2 m x 2 mm Tightspec ID, 4% Carbowax 20M on 80/120 B-DA. The pH of the filtrate was measured with a pH meter (Accumet, model HP-71, Fisher Scientific, Pittsburgh, PA) and ammonia-N was determined using an adaptation for the Technicon auto analyser (Technicon,

Tarrytown, NY) of the Noel and Hambleton (1976) procedure. The adaptation involved colorimetric quantification of N concentration.

Statistical analysis

Data from Experiments 1 and 2 were analysed as completely randomized designs with 2 (control versus enzyme) x 6 (substrates) factorial arrangement of treatments. Data from experiments 3 and 4 were analysed with completely randomized designs with 2 (water versus ruminal fluid) x 6 (substrates) and 2 (control versus enzyme) x 2 (water versus ruminal fluid) x 6 (substrates) factorial treatment arrangements, respectively. The GLIMMIX procedure of SAS (Version 9.2 SAS Institute Inc., Cary, NC) was used to analyse the data. The model used to analyse individual treatment effects included substrate, enzyme treatment, and the interaction (Experiments 1 and 2), substrate and medium, and the interaction (Experiment 3), and substrate, medium, enzyme treatment and all interactions (Experiment 4). Replicate was the random term in each experiment. The Tukey test was used to compare least square means and significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Experiments 1 and 2

The ingredient composition of the concentrates and TMR and the chemical composition of each substrate are shown in Table 1. In general, concentrations of NDF,

Table 1. Ingredient composition of concentrate and total mixed ration (TMR) substrates and chemical composition of all substrates†.

	AH	CS	LC	HC	TMRL	TMRH
Ingredients, % DM						
Corn silage			0	0	49.2	37.0
Alfalfa hay			0	0	13.5	10.0
Cottonseed hulls			12.4	9.4	4.6	5.0
Corn meal			19.8	33.7	7.4	17.9
Citrus pulp			5.4	9.4	2.0	5.0
Whole cottonseed			4.9	9.1	1.8	4.8
SoyPlus[1]			21.2	11.2	7.9	5.9
Soybean meal			6.7	11.3	2.5	6.0
Cottonseed meal			20.9	9.6	7.8	5.1
Mineral mix[2]			8.7	6.1	3.3	3.3
Chemical composition						
DM, %	94.1	35.0	93.9	94.1	64.4	72.2
Ash, % DM	10.7	3.5	10.4	8.1	7.1	6.7
CP, % DM	18.7	9.0	31.1	25.1	18.6	18.5
NDF, % DM	43.6	44.7	27.9	25.4	38.1	33.3
ADF, % DM	23.2	18.4	13.8	12.9	17.9	15.7
Hemicellulose, % DM	20.4	26.2	14.1	12.5	20.2	17.6

[1]West Central Soy, Ralston, IA

[2]Mineral mix contained 26.4% CP, 10.2% Ca, 8.6% Na, 5.1% K, 3.1% Mg, 1.5% S, 0.9% P, 2231 mg/kg of Mn, 1698 mg/kg of Zn, 512 mg/kg of Cu, 339 mg/kg of Fe, 31 mg/kg of Co, 26 mg/kg of I, 7.9 mg/kg of Se, 147,756 IU of vitamin A/kg, 787 IU of vitamin E/kg (DM basis);

†AH= Alfalfa hay; CS= Corn silage; LC=Low concentrate; HC= High concentrate; TMRL=Low concentrate Total Mixed Ration; TMRH=High concentrate Total Mixed Ration.

ADF, and hemicellulose were greater in forages than the TMR and least in concentrates, whereas, WSC concentration was greatest in AH followed by concentrates and least in CS (Table 2). Across all substrates, enzyme treatment increased WSC concentration by 9% and decreased concentrations of NDF, ADF, and hemicellulose by 3.9, 4.1, and 4.3%, respectively. The release of reducing sugars from hydrolyzed cell wall polysaccharides agrees with reported effects of fibrolytic enzymes on various feeds and forages (Hristov *et al.,* 1996; Krause *et al.,* 1998; Krueger and Adesogan, 2008). Greater release of sugars from cell walls could stimulate bacterial glycocalyx production, and thereby increase adhesion of bacteria to substrates (Yang *et al.,* 1999). However, the lack of a substrate x enzyme interaction for most of the chemical measures was unexpected because enzyme-feed specificity is often considered an important determinant of enzyme action (Beauchemin *et al.,* 2004).

Application of the enzyme had substrate-dependent effects on DM disappearance in the buffer (substrate x enzyme interaction, $P<0.001$; Table 3). Enzyme application increased the DMD of HC by 17% but did not affect those of other substrates. Therefore, HC seemed more susceptible to preingestive enzymatic hydrolysis in the buffer than other substrates perhaps because it contained the least NDF concentration. Bowman *et al.* (2002) and Yang *et al.* (2000) also reported that enzyme application to the concentrate portion of the diet of lactating dairy cows improved *in vivo* DMD.

Experiment 3

Incubation of enzyme-treated substrates in RF resulted in greater ($P<0.001$) *in vitro* DMD than incubation in W except for AH, which had similar DMD in both media (substrate x medium interaction; $P<0.001$; Table 4). On average, DMD in RF was 14% greater than DMD in W, confirming that for all feeds except AH, ruminal digestion was more extensive than preingestive disappearance. The similar DMD result for AH in RF and W indicates that preingestive solubility of AH was as extensive as ruminal digestion of the substrate and suggests that digestion of AH in RF was due primarily to solubility rather than enzymatic or microbial hydrolysis. This may be due to the high concentration of water-soluble fractions in alfalfa hay (up to 48.5% of DM; Stefanon *et al.,* 1996).

On average, enzyme-treated substrates incubated in RF had greater ($P<0.001$) NDFD than those incubated in W (Table 4) but the magnitude of the increase was least for AH and greatest for the concentrates (substrate x medium interaction, $P<0.001$). This highlights the greater insolubility of the fiber fractions in the concentrates relative to those in other substrates and illustrates the importance of microbial digestion of such fractions.

Table 2. Effect of enzyme application to different substrates on concentrations of neutral (NDF) and acid (ADF) detergent fiber, hemicellulose, and water-soluble carbohydrates (WSC) (Experiment 1)

Measure	Substrate	Control	Enzyme	Substrate Mean
NDF, % of DM	Alfalfa hay	43.6	42.7	43.2[a]
	Corn silage	44.7	43.4	44.0[a]
	Low concentrate	27.9	25.6	26.8[d]
	High concentrate	25.4	23.4	24.4[e]
	TMRL	38.1	36.2	37.1[b]
	TMRH	33.3	33.0	33.1[c]
	Enzyme treatment mean	35.5[x]	34.1[y]	
	Effects	P values	SEM	
	Substrate	<0.001	0.31	
	Enzyme	<0.001	0.18	
	Substrate x Enzyme	0.22	0.44	
ADF, % of DM	Alfalfa hay	23.2	23.6	23.4[a]
	Corn silage	18.4	17.9	18.2[b]
	Low concentrate	13.8	12.6	13.2[e]
	High concentrate	12.9	11.9	12.4[e]
	TMRL	17.9	16.7	17.3[c]
	TMRH	15.7	15.3	15.5[d]
	Enzyme treatment mean	17.0[x]	16.3[y]	
	Effects	P values	SEM	
	Substrate	<0.001	0.20	
	Enzyme	<0.001	0.11	
	Substrate x Enzyme	0.05	0.28	
Hemicellulose, % of DM	Alfalfa hay	20.4	19.2	19.8[b]
	Corn silage	26.2	25.5	25.9[a]
	Low concentrate	14.1	13.1	13.6[d]
	High concentrate	12.5	11.4	12.0[e]
	TMRL	20.2	19.5	19.9[b]
	TMRH	17.6	17.7	17.6[c]
	Enzyme treatment mean	18.5[x]	17.7[y]	
	Effects	P values	SEM	
	Substrate	<0.001	0.20	
	Enzyme	<0.001	0.12	
	Substrate x Enzyme	0.30	0.29	
WSC, % of DM	Alfalfa hay	9.4	9.8	9.6[a]
	Corn silage	2.2	3.7	2.9[d]
	Low concentrate	7.7	8.8	8.3[b]
	High concentrate	8.4	8.0	8.2[b]
	TMRL	6.0	6.6	6.3[c]
	TMRH	6.1	7.1	6.6[c]
	Enzyme treatment mean	6.7[y]	7.3[x]	
	Effects	P values	SEM	
	Substrate	0.003	0.25	
	Enzyme	<0.001	0.15	
	Substrate x Enzyme	0.16	0.36	

TMRL=Low concentrate Total Mixed Ration; TMRH=High concentrate Total Mixed Ration

[abcde]Means within a column with different superscripts differ, P<0.01

[xy]Means within a row with different superscripts differ, P<0.001.

Table 3. Effect of enzyme application on disappearance of DM from substrates incubated in a buffer for 24 h (Experiment 2)

Substrates	DM disappearance %	
	Control	Enzyme
Alfalfa hay	36.7^{a}	37.1^{a}
Corn silage	20.8^{ef}	19.4^{f}
Low concentrate	26.5^{b}	27.8^{b}
High concentrate	22.0^{de}	25.9^{bc}
TMRL	23.6^{cd}	23.7^{cd}
TMRH	22.9^{de}	22.8^{de}
Enzyme treatment mean	25.4^{y}	26.1^{x}
Effects	P values	SEM
Substrate	<0.001	0.34
Enzyme	0.02	0.20
Substrate x Enzyme	<0.001	0.49

TMRL=Low concentrate Total Mixed Ration; TMRH=High concentrate Total Mixed Ration

^{abcde}Interaction means with different superscripts differ, P<0.001.

^{xy}Means within a row with different superscripts differ, P<0.05.

Table 4. Effect of incubation medium on DMD and NDFD of enzyme-treated substrates (Experiment 3)

Substrates	DMD %		NDFD %	
	Water	Rumen fluid	Water	Rumen fluid
Alfalfa hay	71.4^{de}	73.7^{bcd}	19.6^{d}	26.3^{b}
Corn silage	61.6^{h}	76.5^{b}	6.6^{e}	38.1^{a}
Low concentrate	71.9^{cd}	81.5^{a}	0^{f}	20.0^{cd}
High concentrate	69.1^{ef}	82.5^{a}	2.2^{ef}	26.3^{b}
TMRL	65.3^{g}	72.6^{cd}	3.1^{ef}	36.6^{a}
TMRH	66.4^{fg}	74.8^{bc}	3.5^{ef}	38.9^{a}
Medium mean	67.6^{y}	76.9^{x}	5.8^{y}	30.8^{x}
Effects	P values	SEM	P values	SEM
Substrate	<0.001	0.42	<0.001	0.81
Medium	<0.001	0.24	<0.001	0.47
Substrate x medium	<0.001	0.59	<0.001	1.14

TMRL=Low concentrate Total Mixed Ration; TMRH=High concentrate Total Mixed Ration.

^{abcdef}Means within a measure (DMD or NDFD) with different superscripts differ, P<0.001.

^{xy}Means within a row with different superscripts differ, P<0.001.

Since untreated controls were not included in this experiment, it is not clear if the greater DMD and NDFD in RF versus W reflect the intrinsic digestibility of the substrates or synergy between the exogenous enzyme and ruminal microbes in RF (Morgavi *et al.,* 2000). This aspect was further explored in Experiment 4, which compared untreated and enzyme-treated substrates in both media.

Experiment 4

Incubation in RF resulted in greater DMD and NDFD than incubation in W but the magnitude of the responses differed with substrate type (substrate x medium interaction; P<0.001 and=0.0017, respectively; Table 5 and 6). Averaged across

control and enzyme treatment, increases in DMD of 12, 17, 7, 7, 16, and 13% occurred when AH, CS, LC, HC, TMRL, and TMRH were incubated in RF instead of W, respectively. Therefore, incubation medium had the greatest effect on the DMD of CS and the least on LC and HC.

Enzyme effects on DMD and NDFD depended on the incubation medium (P=0.002 and 0.066, respectively) suggesting that preingestive and ruminal effects of the enzyme differed. Averaged across substrates, enzyme addition increased DMD in W (66.8 vs. 69.2%) by 4% but had no effect on DMD in RF (76.0 vs. 76.1%). Enzyme treatment increased NDFD in W (12.5 vs. 8.9%) by about 40% but did not affect NDFD in RF (30.7 vs. 31.7%). These results suggest that applying the enzyme contributed more to preingestive hydrolysis of the substrates than to their digestion in ruminal fluid and indicates that the greater DMD and NDFD in RF versus W in Experiment 3 reflects the intrinsic digestibility of the substrates rather than synergy between the exogenous enzyme and ruminal microbes in RF (Morgavi *et al.,* 2000). This preingestive enzyme effect on DMD and NDFD explains some of the enzyme-mediated increases in *in vivo* apparent DMD, NDFD, and efficiency of milk production in the previous study (Arriola *et al.,* 2011). That enzymic hydrolysis increased the DMD of only HC after 24 h of incubation in the buffer (Table 3) but increased those of all substrates after 24 h of incubation in the buffer followed by 24 h of incubation in W (Table 5), suggests that the initial 24 h incubation in the buffer was too short to demonstrate preingestive effects of the enzyme on all substrates.

Table 5. Effect of enzyme application and incubation medium on DMD of substrates incubated for 24 h in water or ruminal fluid after incubation for 24 h in a buffer (Experiment 4)

Substrates	Medium					
	Water			Rumen fluid		
	Control	Enzyme	Mean	Control	Enzyme	Mean
Alfalfa hay	59.2	61.2	60.2^{f}	67.4	67.6	67.5^{e}
Corn silage	58.0	61.3	59.6^{f}	69.8	69.9	69.9^{d}
Low concentrate	74.4	77.5	75.9^{c}	80.8	81.6	81.2^{ab}
High concentrate	76.4	78.5	77.4^{c}	82.4	83.6	83.0^{a}
TMRL	64.6	66.4	65.5^{e}	76.5	75.9	76.2^{c}
TMRH	68.3	70.1	69.2^{d}	78.9	77.9	78.4^{bc}
Mean	66.8^{y}	69.2^{x}		76.0	76.1	
Effects	P values	SEM				
Substrate	<0.001	0.43				
Enzyme	0.0007	0.25				
Medium	<0.001	0.35				
Substrate x Medium	<0.001	0.60				
Enzyme x Medium	0.0022	0.35				
Substrate x Enzyme	0.733	0.60				
Substrate x Enzyme x Medium	0.939	0.85				

TMRL=Low concentrate Total Mixed Ration; TMRH=High concentrate Total Mixed Ration.

^{abcdefg}Substrate x medium means with different superscripts differ, P<0.001.

^{xy}Enzyme x medium means with different superscripts differ, P<0.01.

Table 6. Effect of enzyme application and incubation medium on NDFD of substrates of substrates incubated for 24 h in water or ruminal fluid after incubation for 24 h in a buffer (Experiment 4)

Substrates	NDFD					
	Water			Rumen fluid		
	Control	Enzyme	Mean	Control	Enzyme	Mean
Alfalfa hay	6.1	10.7	8.4^{e}	25.0	25.4	25.2^{bc}
Corn silage	2.5	10.3	6.4^{e}	30.0	30.1	30.1^{b}
Low concentrate	0.0	0.0	0.0^{f}	14.3	17.8	16.1^{d}
High concentrate	0.0	0.0	0.0^{f}	17.6	23.6	20.6^{cd}
TMRL	18.5	22.7	20.6^{cd}	46.0	44.7	45.3^{a}
TMRH	26.4	30.6	28.5^{b}	50.9	48.8	49.8^{a}
Mean	8.9^{y}	12.2^{x}		30.7	31.7	

Effects	P values	SEM
Substrate	<0.001	0.83
Enzyme	0.0011	0.48
Medium	<0.001	0.48
Substrate x Medium	0.0018	1.17
Enzyme x Medium	0.0662	0.68
Substrate x Enzyme	0.7814	1.17
Substrate x Enzyme x Medium	0.0304	1.65

TMRL= Low concentrate Total Mixed Ration; TMRH=High concentrate Total Mixed Ration.

^{abcde}Substrate x medium means with different superscripts differ, $P<0.001$.

^{xy}Enzyme x medium means with different superscripts differ, $P<0.001$.

Reasons why enzyme effects on DMD and NDFD in RF were not apparent are unknown. Many other studies evaluating enzyme effects on DMD of dairy cattle feeds have used 24 h ruminal fluid incubation durations to demonstrate positive enzyme effects (Eun and Beauchemin, 2007; Eun *et al.,* 2007; Krueger and Adesogan, 2008). Nevertheless, greater enzyme effects on DMD and NDFD may have occurred if substrates were incubated for 30 (Oba and Allen, 1999) or 48 h (NRC, 2001) instead of 24 h to reflect ruminal NDF retention times in dairy cattle. Factors other than the duration of ruminal digestion also may be involved. For instance, fibrolytic enzyme application decreased ruminal NDFD of a TMR fed to dairy cows but increased postruminal NDFD for unknown reasons (Sutton *et al.,* 2003).

Ruminal pH and concentrations of total VFA and ammonia-N differed ($P<0.001$) with substrate type (Table 7). Ruminal pH was greatest for AH and least for CS, possibly reflecting the differences in the starch concentrations of the substrates, as well as their ammonia-N concentration and buffering capacity. Total VFA concentrations were less in forages than TMR and butyrate molar proportion was greatest for CS and least for AH. Ammonia-N concentration was least for CS and greatest for LC, partly due to their different CP concentrations.

Table 7. Effect of enzyme (Enz) application on pH, VFA and ammonia-N concentrations of substrates incubated for 24 h in ruminal fluid after incubation for 24 h in a buffer (Experiment 4)

Substrate		pH	Total VFA, mM	Acetate (A), mol/ 100 mol	Propionate (P), mol/ 100 mol	Butyrate, mol/100 mol	A:P	Ammonia-N, mg/L
AH	Control	7.17	135	57.6	32.0	8.9	1.93	58.3
	Enz	7.16	165	67.3	24.4	7.1	2.76	60.4
	Mean	7.17[a]	150[d]	62.4	28.2	8.0[c]	2.3	59.3[b]
CS	Control	6.01	160	57.4	28.4	13.2	2.03	30.1
	Enz	5.91	194	57.3	28.4	13.2	2.02	26.3
	Mean	5.96[d]	177[c]	57.3	28.4	13.2[a]	2.02	28.2[d]
LC	Control	7.15	178	60.4	28.2	10.3	2.15	76.0
	Enz	6.91	180	59.3	29.0	10.6	2.05	75.9
	Mean	7.03[ab]	179[bc]	59.8	28.6	10.5[b]	2.10	75.9[a]
HC	Control	6.71	184	58.2	30.4	10.4	1.92	65.9
	Enz	6.72	190	57.6	30.4	11.0	1.90	60.8
	Mean	6.72[bc]	187[abc]	57.9	30.4	10.7[b]	1.91	63.3[b]
TMRL	Control	6.44	205	60.3	28.1	10.6	2.15	53.1
	Enz	6.51	216	60.5	27.7	10.8	2.19	49.4
	Mean	6.47[c]	211[a]	60.4	27.9	10.7[b]	2.17	51.3[c]
TMRH	Control	6.43	192	59.4	28.4	11.2	2.10	52.6
	Enz	6.42	221	59.8	28.5	10.8	2.10	48.3
	Mean	6.43[c]	207[ab]	59.6	28.4	11.0[b]	2.10	50.4[c]
SEM[1]		0.10	9.18	1.89	1.44	0.42	0.15	1.55
Effect					*P* values			
Substrate		<0.001	<0.001	0.13	0.58	<0.001	0.16	<0.001
Enzyme		0.45	0.002[2]	0.20	0.16	0.45	0.18	0.01[2]
Substrate x Enzyme		0.73	0.23	0.08	0.07	0.11	0.05	0.18

AH= Alfalfa hay; CS= Corn silage; LC= Low energy concentrate; HC= High energy concentrate; TMRL= Low concentrate Total Mixed Ration; TMRH = High concentrate Total Mixed Ration

[a,b,c,d]Means in the same column with different superscripts differ, $P < 0.001$

[1]Main enzyme effect means for total VFA were 176 versus 194 mM, SEM = 3.48 and were 56 versus 54 mg/L, SEM= 0.63 for ammonia-N.

Enzyme addition increased (P=0.002) total VFA concentration (194 vs. 176 Mm) suggesting that the enzyme increased supply of energy yielding substrates from fermentation of the dietary substrates. Enzyme treatment also decreased (P<0.01) ruminal ammonia-N concentration (56 vs. 54 mg/L), which may reflect greater utilization of ammonia-N for microbial growth due to increased availability of WSC during ruminal fermentation. Effects of enzyme addition on the molar proportions of acetate and propionate tended to differ with the substrate. Compared to control, enzyme-treated AH had greater molar proportion of acetate (P=0.08) and lower proportion of propionate (P=0.07) resulting in a greater (P=0.05) acetate to propionate ratio, but such differences did not occur in other substrates. Eun and Beauchemin (2007) reported that adding the same enzyme to alfalfa hay increased total VFA concentration, propionate proportion, and NDFD, but did not affect acetate to propionate ratio. Whereas adding the enzyme to corn silage decreased acetate and propionate proportions and the acetate to propionate ratio but did not affect total VFA concentration. Differences between their results and those in this study may be due to differences between the forages and ruminal fluid activity as well as procedural differences for estimating these parameters.

Beneficial enzyme effects on concentrations of cell walls and reducing sugars, digestibility of DM or NDF in W or RF, or ruminal fluid VFA or ammonia-N concentrations did not ($P > 0.05$) depend on the substrate. This indicates that none of the substrates was preferentially hydrolyzed by the enzyme in RF or W and contradicts the hypothesis that enzyme effects on the forages would be greater. Therefore, it is unlikely that beneficial enzyme effects on DMD, NDFD, and efficiency of milk production in the study of Arriola *et al.* (2011) were due to preferential hydrolysis of the concentrate or either of the forages in the diets. Rather, the enzyme probably affected the entire TMR. Effects of enzyme addition to specific portions of diets have been contradictory. Yang *et al.* (2000) compared treating either the TMR or concentrate with an enzyme and reported that improvements in DMD but not NDFD tended to be greater when the concentrate was treated and only concentrate treatment improved milk yield. In contrast, Phipps *et al.* (2000) reported no differences between milk yield of cows fed enzyme-treated concentrates or TMR or the untreated TMR. Sutton *et al.* (2003) also reported no differences in DMI or milk yield of cows fed diets in which an enzyme was infused ruminally or added to the TMR or concentrate. These contradictions and the results of this study do not refute the existence of enzyme-feed specificity (Beauchemin *et al.,* 2004) and its' importance in determining enzyme effects. Rather, because the substrates, diets, and ingredients evaluated in this and other studies are comprised of various types and proportions of cell walls and other chemical components, they are probably not homogenous enough to reflect enzyme-feed specificity. Therefore, continued research on the best portion of the diet to which enzymes should be added may not identify an ideal ingredient target for all diets.

CONCLUSION

Enzyme treatment decreased ruminal ammonia-N concentration and increased hydrolysis of cell walls, release of reducing sugars, digestibility of DM and NDF, and total VFA concentration regardless of substrate type. Therefore, this study provided no convincing evidence that the enzyme preferentially hydrolyzed specific dietary substrates. Substrate digestion in RF was consistently greater than that in W. However, enzyme effects on DMD and NDFD were consistently greater in W than in RF, indicating that preingestive effects of the enzyme were greater than ruminal effects under the conditions of the study. Preingestive effects were also greater for AH than other substrates, likely reflecting the high concentration of water-soluble fractions in AH. These experiments involved 24 h incubations in RF or W; therefore future research should investigate whether similar results are obtained with longer incubation periods.

REFERENCES

Arriola, K.G., Kim, S.C., Staples, C.R. and Adesogan, A.T. 2011. Effect of fibrolytic enzyme application to low and high-concentrate diets on the performance of lactating dairy cattle. *Journal of Dairy Science,* 94: 832-841.

Bailey, M.J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology,* 23: 257-270.

Beauchemin, K.A., Yang, W.Z. and Rode, L.M. 1999. Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. *Journal of Dairy Science,* 83: 378-390.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science,* 81 (E. Suppl. 2): E37-E47

Beauchemin, K.A., Colombatto, D. and Morgavi, D.P. 2004. A rationale for the development of feed enzyme products for ruminants. *Canadian Journal of Animal Science,* 84: 23-36.

Bowman, G.R., Beauchemin, K.A. and Shelford, J.A. 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. *Journal of Dairy Science,* 85: 3420-3429.

Elwakeel, E.A., Titgemeyer, E.C., Johnson, B.J., Armendariz, C.K. and Shirley, J.E. 2007. Fibrolytic enzymes to increase the nutritive value of dairy feedstuffs. *Journal of Dairy Science,* 90: 5226-5236.

Eun, J.S. and Beauchemin, K.A. 2007. Enhancing *in vitro* degradation of alfalfa hay and corn silage using feed enzymes. *Journal of Dairy Science,* 90: 2839-2851.

Eun, J.S., Beauchemin, K.A. and Schulze, H. 2007. Use of exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. *Journal of Dairy Science,* 90: 1440-1451.

Feng, P., Hunt, C.W., Pritchard, G.T. and Julien, W.E. 1996. Effect of enzyme preparations on *in situ* and *in vitro* degradation and *in vivo* digestive characteristics of mature cool-season grass forage in beef steers. *Journal of Animal Science,* 74: 1349-1357.

Fontes, C.M.G.A., Hall, J., Hirst, B.H., Hazlewood, G.P. and Gilbert, H.J. 1995. The resistance of cellulases and xylanases to proteolytic inactivation. *Applied Microbial Biotechnology,* 43: 52-57.

Hall, M.B., Hoover, W.H, Jennings, J.P. and Webster, T.K.M. 1999. A method for partitioning neutral detergent-soluble carbohydrates. *Journal of the Science of Food and Agriculture,* 79: 2079-2086.

Hristov, A.N., Rode, L.M, Beauchemin, K.A. and Wuerfel, R.L. 1996. Effect of a commercial enzyme preparation on barley silage *in vitro* and in sacco dry matter degradability. *Proceedings of the Western Section Meeting of the American Society of Animal Sciences,* 47: 282-284.

Hristov, A.N., McAllister, T.A. and Cheng, K.J. 2000. Intraruminal supplementation with increasing levels of exogenous polysaccharide-degrading enzymes: Effects on nutrient digestion in cattle fed a barley grain diet. *Journal of Animal Science,* 78: 477-487.

Krause, M., Beauchemin, K.A, Rode, L.M, Farr, B.I. and Nørgaard, P. 1998. Fibrolytic enzyme treatment of barley grain and source of forage in high-grain diets fed to growing cattle. *Journal of Animal Science,* 76: 2912-2920.

Krueger, N.A. and Adesogan, A.T. 2008. Effects of different mixtures of fibrolytic enzymes on digestion and fermentation of bahiagrass hay. *Animal Feed Science and Technology,* 145: 84-94.

Krueger, N.A, Adesogan, A.T., Staples, C.R, Krueger, W.K, Kim, S.C, Littell, R.C. and Sollenberger, L.E. 2008. Effect of method of applying enzymes or ammonia to bermudagrass hay on feed intake, digestion, and growth of beef steers. *Journal of Animal Science,* 86: 882-889.

Kung, L.Jr., Treacher, R.J, Nauman, G.A, Smagala, A.A, Endres, K.M. and Cohen, M.A. 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. *Journal of Dairy Science,* 83: 115-122.

Lewis, G.E., Hunt, C.E, Sanchez, W.K, Treacher, R., Pritchard, G.T. and Feng, P. 1996. Effect of direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. *Journal of Animal Science,* 74: 3020-3028.

Lewis, G.E., Sanchez, W.K., Hunt, C.W, Guy, M.A., Pritchard, G.T., Swanson, B.I. and Treacher, R.J. 1999. Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. *Journal of Dairy Science,* 82: 611-617.

Mastihuba, V., Kremnicky, L., Mastihubova, M., Willet, J.L. and Cote, G.L. 2002. A spectrophotometric assay for feruloyl esterases. *Analytical Biochemistry,* 309: 96-101.

McAllister, T.A., Oosting, S.J., Popps, J.D., Mir, Z., Yanke, L.J., Histrov, A.N., Treacher, R.J. and Cheng, K.J. 1999. Effects of exogenous enzymes on digestibility of barley silage and growth performance of feedlot cattle. *Canadian Journal of Animal Science,* 79: 353-360.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., Iwaasa, A.D., Yang, W.Z., McAllister, T.A. and Wang, Y. 2000. Synergistic between ruminal fibrolytic enzymes and enzymes from *Trichoderma longibrachiatum*. *Journal of Dairy Science,* 83: 1310-1321.

Noel, R.J. and Hambleton, L.G. 1976. Collaborative study of a semi automated method for determination of crude protein in animal feeds. *Journal of the Association of Official Analytical Chemists,* 59: 134-140.

NRC. 2001. *Nutrient Requirements of Dairy Cattle*, 7th rev. ed. National Academy of Science. Washington, DC.

Oba, M. and Allen, M.S. 1999. Effects of brown midrib 3 mutation in corn silage on dry matter intake and productivity of high yielding dairy cows. *Journal of Dairy Science,* 82: 135-142.

Phipps, R.H., Sutton, J.D., Beever, D.E., Bhat, M.K., Hartnell, G.F., Vicini, J. and Hard, D.L. 2000. Effect of cell-wall degrading enzymes and method of application on feed intake and milk production of Holstein-Friesian dairy cows. *Journal of Dairy Science,* 83(Suppl. 1): 236-237 (Abstr.).

Rode, L.M., Yang, W.Z. and Beauchemin, K.A. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. *Journal of Dairy Science,* 82: 2121-2126.

SAS Institute. 2002. *SAS User's Guide. SAS Institute*. Inc., Cary, NC.

Stefanon, B., Pell, A.N. and Schofield, P. 1996. Effect of maturity on digestion kinetics of water-soluble and water-insoluble fractions of alfalfa and brome hay. *Journal of Animal Science,* 74: 1104-1115.

Sutton, J.D., Phipps, R.H., Beever, D.E., Humphries, D.J., Hartnell, G.F., Vicini, J.L. and Hard, D.L. 2003. Effect of method of application of a fibrolytic enzyme product on digestive processes and milk production in Holstein-Friesian cows. *Journal of Dairy Science,* 86: 546-556.

Tilley, J.M.A. and Terry, R.A. 1963. A two stage technique for the *in vitro* digestion of forage crops. *Journal of British Grassland Society,* 18: 104-111.

Treacher, R, McAllister, T.A., Popp, J.D., Mir, Z., Mir, P. and Cheng, K.J. 1997. Effects of exogenous cellulases and xylanases on feed utilization and growth performance of feedlot steers. *Canadian Journal of Animal Science,* 77(Suppl.): 541.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3568-3597.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 1999. Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. *Journal of Dairy Science,* 82: 391-403.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 2000. A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science,* 83: 2512-2520.

Wood, T.M. and Bhat, M.K. 1988. Methods for measuring cellulase activities. In: *Methods in Enzymology* (Eds. W.A. Wood and S.T. Kellog), Vol. 160. Academic Press Inc., London, UK. pp. 87-112.

Animal Nutrition and Feed Technology (2013) 13: 551-568

Response of Different Tropical Pasture Grass Species to Treatments with Fibrolytic Enzymes in Terms of *In Vitro* Ruminal Nutrient Degradation and Methanogenesis[#]

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ABSTRACT

Soltan, Y.A., Abdalla, A.L., Silva, L.R.F., Natel, A.S., Morsy, A.S. and Louvandini, H. 2013. Response of different tropical pasture grass species to treatments with fibrolytic enzymes in terms of in vitro ruminal nutrient degradation and methanogenesis. Animal Nutrition and Feed Technology, 13: 551-568.

Two fibrolytic enzyme products (cellulase and xylanase) were applied at 7.5 or 0.46 enzymatic units/500 mg DM substrate, respectively to common Brazilian forage pasture grass species: Aruana (*Panicum maximum*), Napier (*Pennisetum purpureum*), Brachiaria (*Brachiaria decumbens*), Buffel (*Pennisetum ciliare*), Sugarcane (*Saccharum officinarum)* and sugarcane bagasse. The forages were then incubated in an *in vitro* semi-automatic gas production (GP) system for 24 and 48 h. Cellulase addition enhanced ($P<0.05$) GP, the amount of degraded neutral detergent fiber (DNDF), and propionate and butyrate production during 24 h compared with no addition, whereas xylanase product had only minor effects on nutrient degradation. None of the enzymatic treatments affected methane (CH_4) at the two incubation times. Aruana and Napier had the highest hemicellulose and protein contents, caused the lowest CH_4 production ($P<0.01$) and increased ($P<0.05$) GP, DNDF and CH_4 at 24 h when treated with cellulase product compared with the other grasses. The present results highlighted the importance of matching the enzyme product to forage substrate properties to achieve maximal benefit from exogenous enzyme application in ruminant nutrition.

Key words: Endoglucanase, Methane, Pasture quality, Sustainability, Xylanase

INTRODUCTION

Beef and dairy cattle production play an important role in the Brazilian economy where the predominant livestock production systems are based mostly on grazing and rely on native pastures and cultivated grass pastures. A major constraint is that, less

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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than 50% of the cell wall components (the main nutrient) of most tropical forage grasses are readily digested and utilized (Abdalla *et al.*, 2012b). This results excessive nutrient excretion, low intake of digestible energy and high methane (CH_4) production which represent a significant loss of dietary energy that could potentially be redirected towards the production of milk or meat rather than contributing to climate change as a greenhouse gas (Eckard *et al.*, 2010; Goel and Makkar, 2012; Soltan *et al.*, 2012). To date, various CH_4 mitigation strategies have been applied. However, it appears that CH_4 reduction is often associated with a decrease in feed digestibility. Additionally, many of these approaches are investigated only over short periods, but may get inactive by microbial adaptation (Zhou *et al.*, 2011; Abdalla *et al.*, 2012a; Goel and Makkar, 2012).

Exogenous feed enzymes products that contain primarily xylanolytic and cellulolytic activities may enhance rumen fiber digestion, and improve feed conversion efficiency and thus lead to enhanced productive efficiency of ruminants (Holtshausen *et al.*, 2011). However, the manufactures' recommended doses of most commercial fibrolytic enzyme products have been determined under conditions most favorable for ranges of pH (4.2 to 6.5) and temperatures (40 to 57°C) which are not always close to ruminal conditions. Thus the random addition of these products to ruminant diets without consideration for specific rumen conditions (pH 6.0 to 6.5 and 39°C) and the not yet tested efficiency for certain substrate will result in unpredictable effects and thus discourage the adoption of the enzyme technology (Beauchemin *et al.*, 2003; Colombatto and Beauchemin, 2003; Holtshausen *et al.*, 2011).

Hydrogen is a major intermediary metabolite in the ruminal degradation of organic matter, that is mainly used by methanogenic *Archaea* to reduce carbon dioxide into methane. Thus theoretically, enhancing the fiber degradability by addition of exogenous enzyme will promote CH_4 emissions as well. However, when expressed per unit of animal-source feed produced, this will be different as the expected improvement in fiber digestibility and ruminal fermentation would improve feed conversion efficiency and overall animal productivity at the same time. In order to approach towards an answer to this hypothesis, the effect of two different fibrolytic enzymes added at levels following manufacturers' recommendations at ruminal conditions was determined *in vitro* in a large number of common tropical pasture grass species in comparison to incubations without enzymes. The focus was put on ruminal nutrient degradability, fermentation products and methanogenesis.

MATERIALS AND METHODS

The study was carried out at the Center for Nuclear Energy in Agriculture, University of Sao Paulo (CENA/USP), Piracicaba, Brazil. The animals donating rumen fluid were always treated in accordance to the guidelines of the Internal Commission for Environmental and Ethics in Experimentation with Animals of CENA/USP.

Substrates

About 20 kg of freshly chopped samples of the most common Brazilian pasture grass species, i.e. Aruana (*Panicum maximum;* n=20), Brachiaria (*Brachiaria decumbens;* n=14), Napier grass (*Pennisetum purpureum*; n=14), Buffel grass (*Pennisetum ciliare*; n=4) and Sugarcane (*Saccharum officinarum;* n=4*),* were harvested between 08.00 and 10.00 h from 0.25m^2 plots from different production sites at Piracicaba, Sao Paulo state- Brazil. Additionally sugarcane bagasse (n=4) was included. The samples were harvested with scissors at a height of 20 cm (i.e., the grazing portion of the stand). Grasses samples were collected during the entire year 2011 at different regrowth periods (2, 3 or 4 weeks) according to their availability. The plant samples were subjected to 72 h oven drying at 40°C before being milled through 1 mm screen. The individual grasses were chemically characterized and then, pooled individually by browse species in equal proportional fractions (10% of each collection) for the *in vitro* enzyme assay.

Chemical analysis

Forage samples (pooled as well as the individual plant samples) were analysed according to AOAC (1995) for dry matter (DM),organic matter (OM), crude protein (CP, as 6.25×N), and ether extract (EE). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin were measured sequentially using the Ankom Fiber Analyzer and filter Ankom bags. They were expressed exclusive of residual ash according to Van Soest *et al.* (1991) and adapted to Mertens (2002). The NDF was assayed with a heat-stable amylase. Acid detergent lignin (ADL) was determined by solubilization of cellulose with sulfuric acid (72%) according to Van Soest *et al.* (1991). Hemicellulose and cellulose were calculated as the differences between NDF and ADF, and ADF and ADL, respectively.

Enzyme products and characterization of their enzymatic activities

Two exogenous fibrolytic enzymes were tested: Cellulase (Dyadic Cellulase PLUS) product and xylanase (Dyadic xylanase PLUS) product fermented by *Trichoderma longibrachiatum* and commercialized by Dyadic International, Inc.. (Florida, USA). The enzyme products were assayed for endoglucanase (EC3.2.1.4.), and exoglucanase (EC3.2.1.91) activities following the procedures described by Wood and Bhat (1988) and for xylanase (EC3.2.1.8.) activity according to Bailey *et al.* (1992). All activities were measured at pH 6.5 and 39°C in order to simulate optimal rumen conditions (Colombatto and Beauchemin, 2003; Holtshausen *et al.*, 2011).

One enzymatic unit was defined as the amount of enzyme required to release 1 μmol of xylose or glucose/min from the corresponding substrate at 39°C and pH 6.5 (Giraldo *et al.*, 2008). One ml of the cellulase product liberated 277 μmol of xylose/min from oat spelt xylan, 720 μmol of glucose/min from carboxymethylcellulose and 1.17 μmol of glucose/min from SIGMACELL cellulose; while one ml of the xylanase

product liberated 6760 μmol/min of xylose from oat spelt xylan, 732 μmol of glucose/min from carboxymethylcellulose and 0 μmol of glucose/min from SIGMACELL cellulose.

Enzyme treatments

Half gram of the individual dried and ground substrates was weighted into pre-weighted dry artificial filter bag (#F57 bags; 50 X 40 mm; 25±10 microns pore size; ANKOM Technology Corporation, Macedon, NY, USA) then it was put in a glass bottle of total volume of 160 ml. The enzyme preparations were added at the manufacture recommended dose as 7.5 endoglucanase or 0.46 xylanase unit/500 mg DM substrate from the cellulase and xylanase products, respectively. Control samples received no enzyme addition. The enzyme solutions were prepared by dissolving the enzyme products with Menke's buffered medium designed for gas production (GP) tests (Longo *et al.*, 2006) to reach concentrations of 7.5 or 0.46 units per ml buffer of endoglucanase and xylanase, respectively. One ml of Menke's buffer (non-supplemented controls) or the enzyme solution was combined directly with each corresponding substrate contained in the bag and was kept at room temperature (21-23°C) for 18 h before starting the GP assay (Elwakeel *et al.*, 2007). This was done because of the importance of adsorption and binding of the enzyme to substrate before feeding to allow proper attachment and protection against degradation by rumen proteases. All incubations were performed in duplicated in three runs using different rumen inocula and at two incubation times, 24 and 48 h, with the latter considered to bethe end point of the substrate degradation (Beauchemin *et al.*, 2003).

Inoculum donors and procedures

Nine adult rumen-cannulated Santa Inês sheep (60±2.5 kg body weight) grazing a tropical grass pasture *(Tifton 85; Cynodon* spp.) and supplemented with ground maize and soybean meal (0.7 kg/100 kg live weight, 14% crude protein) with free access to a mineral premix and fresh water were used as inoculum donors. Ruminal liquid and solid fractions were collected separately from each animal before the morning feeding and kept in pre-warmed thermo containers (39°C) under anaerobic conditions. A liquid fraction was obtained by using a stainless steel probe (2.5 mm screen) attached to a syringe with large capacity. Similar volumes (1:1 v/v) of both fractions were blended for 10 s, squeezed through three layers of cheesecloth, and maintained in a water bath (39°C) under CO_2 until inoculation took place (Bueno *et al.*, 2005). Each treatment was incubated in three inocula, each prepared using three animals per inoculum. The different inocula were used to avoid the individual animal effect. In each inoculum, four bottles were prepared for each substrate sample: two were incubated for 24 h and the other two for 48 h. The same system was used for the blank (flasks without substrate containing inoculum+buffer medium) to obtain the net GP and CH_4; an internal standard (Tifton hay; *Tifton-85 Cynodon* sp.) was included for each inoculum to enable adjustments among inocula. Biased incubation inocula (variations above 10%) were rejected.

In vitro gas and methane production

The *in vitro* GP assay (Theodorou *et al.*, 1994) was adapted to a semi-automatic system (Bueno *et al.*, 2005) using a pressure transducer and a data logger (Pressure Press Data 800, LANA, CENA/USP, Piracicaba, Brazil). To each glass bottle that contained the sample bag, 50 ml of incubation medium (Menke's buffered medium) and 25 ml of rumen inoculum were added to obtain a head space of 85 ml of the total volume of 160 ml. Bottles were sealed immediately with 20 mm butyl septum stoppers (Bellco Glass Inc., Vineland, NY, USA), manually mixed, and incubated at 39°C in a forced air oven (Marconi MA35, Piracicaba, SP, Brazil) either for 24 or 48 h. Head space gas pressure was measured at 4, 8, 12, 24 and, where available, 48 h. Gas production was calculated as $V=7.365\times P$, where, 7.365 is the regression slope, V is gas volume (ml) and P is the pressure (psi) measured (Soltan *et al.*, 2012).

For CH_4 determination, 2.5 and 2 ml of gas were sampled from the bottles after 24 and 48 h incubation, respectively using a 5 ml syringe (Becton Dickson Indústria Cirúrgica LTDA, Curitiba, Brazil) and stored in a 10 ml vacuum tube. After each gas sampling, bottles were vented, mixed, and returned to the incubator. The CH_4 concentration was determined using a gas chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD, 1.0 mm ID, 1 m length; Ref. no 19809; Restek, Bellefonte, PA, USA) using an external calibration analytical curve (0, 30, 60, 90 and 120; ml/l) prepared with pure CH_4 (White Martins PRAXAIR Gases industrial Inc., Osasco, Brazil; 995 ml/l purity). The temperatures of the column, injector, and flame ionization detector were 60°C, 200°C, and 240°C, respectively. Helium at 10 ml/min was used as the carrier gas. The CH_4 concentration was determined according to Longo *et al.* (2006) as CH_4, ml= (total gas, ml + headspace, 85 ml)$\times CH_4$ concentration, ml/ml. Net CH_4 was expressed as ml/g TDOM and ml/g DNDF calculated by correcting for the corresponding blank.

Rumen degradability and fermentation characteristics

The truly degraded organic matter (TDOM) was determined according to Van Soest *et al.* (1991) by neutral detergent solution (NDS) treatment but with some modifications. After termination of the incubation (24 or 48 h), all filter bags that contained the residual matter of the sample were removed and were put immediately on ice to stop the microbial fermentation process. All bags were treated with NDS for 1 h at 90°C with heat stable α-amylase, then washed with hot water and finally with acetone. The DM and ash contents of the residual matter were determined. The difference between the incubated OM amounts incubated and remaining not degraded was considered to be TDOM, and the difference between the amount of NDF incubated and remaining and residual NDF was calculated as the degraded NDF (DNDF). The partitioning factor, an index of microbial synthesis efficiency (PF) was calculated with as the ratio of TDOM (mg) and gas volume (ml) (Blümmel *et al.*, 1997).

The contents of the bottles were used for determining fermentation characteristics. The net release values of NH_3-N were measured according to Preston (1995). Short-chain fatty acids (SCFA) were determined according to Palmquist and Conrad (1971). Rumen fluid was centrifuged (15.000 *g* for 10 min at 4°C) to remove large feed particles; 1.6 ml of the supernatant was centrifuged (15.000 *g* for 15 min at 4°C) after adding 0.4 ml solution of metaphosphoric acid (3: 1) 25% and formic acid 98%-100% plus 0.2 ml of 2-ethyl-butyric acid 100 mM (internal standard, MW=116.16; Sigma Chemie Gmbh, Steinheim, Germany). After centrifugation, approximately 1.2 ml was transferred to the chromatographic vial. One µl was injected into the gas chromatograph (GC HP 5890 Series II/integrator HP 3396 Series II/ automatic injector HP 6890 Series, Agilent Technologies, Palo Alto, CA, USA). A mixture of SCFA with known concentrations was used as the external standard for integrator calibration.

Statistical analysis

The data were subjected to analysis of variance (ANOVA), using the General Linear Model procedure (GLM) of SAS software (SAS, 2002). The experimental unit of substrate chemical analysis was the composite sample of each forage species and numbers of sample collections for the same plant were considered as repetitions. The enzymatic effects were statistically analysed separately for each activity and incubation time (6 substrates, 3 inocula and 2 enzymes levels) and analysed with a model that included substrate, enzyme level (with or without additive), their interaction as fixed effects, and experimental run (replication in time) as a random effect. Data were also analysed separately for each substrate and incubation time and all means were compared using the Tukey test. The improvements in TDOM and DNDF due to the enzymes addition (i,e., the difference in TDOM or DNDF between the same substrate with and without enzyme addition in relation to TDOM or DNDF without enzyme addition) were analysed by a paired t-test for all substrates and the individual substrates. All significances were declared at $P<0.05$.

RESULTS

Chemical composition of the experimental grasses

There were wide variations in the chemical composition of the grass samples investigated (Table 1). Aruana, Napier and Brachiaria presented the highest ($P<0.01$) CP content while Buffel, Sugarcane and bagasse showed lower values (161, 156, 105, 77, 36 and 34 g/kg DM, respectively). Buffel and sugarcane bagasse had the highest ($P<0.01$) concentrations of NDF (792 and 741 g/kg DM), ADF (484 and 513 g/kg DM) and cellulose (409 and 414 g/kg DM) compared with the other substrates (628, 384 and 308 g/kg DM for NDF, ADF and cellulose, respectively) while sugarcane showed the lowest ($P<0.01$) hemicellulose concentrations. A similar trend for the fiber composition was found in the individual pooled samples compared with the individual

samples for each grass. Values for the pooled samples of Aruana, Brachiaria, Napier, Buffel, sugarcane bagasse and sugarcane were 713, 692, 592, 796, 835 and 529 g NDF/kg DM, 449, 388, 367, 500, 558 and 341 g ADF/kg DM and 83, 65, 51, 84, 99 and 54 g lignin/kg DM, respectively.

Table 1. Chemical composition (g/kg DM) of the grass samples plus sugarcane and bagass harvested in the growing season

Attributes	Grasses						SEM
	Aruana (n=20)	Brachiaria (n=14)	Napier (n=14)	Buffel (n=4)	Sugarcane bagasse (n=4)	Sugarcane (n=4)	
Organic matter	889^{bc}	908^{b}	856^{c}	923^{b}	974^{a}	982^{a}	30.4
Crude protein	161^{a}	105^{bc}	156^{ab}	77^{cd}	34.2^{d}	31.5^{d}	35.7
Neutral detergent fiber	704^{bc}	665^{c}	606^{d}	792^{a}	741^{ab}	537^{e}	40.5
Acid detergent fiber	422^{b}	380^{bc}	374^{c}	484^{a}	513^{a}	360^{c}	30.2
Lignin	78^{b}	62^{b}	59^{b}	74^{b}	99^{a}	71^{b}	14.8
Hemicellulose	281^{a}	285^{a}	232^{b}	309^{a}	228^{b}	176^{c}	30.9
Cellulose	344^{b}	319^{bc}	315^{bc}	409^{a}	414^{a}	289^{c}	30.6
NDIN	19.9^{a}	8.6^{b}	16.6^{a}	6.1^{b}	2.7^{b}	3.1^{b}	4.32
ADIN	13.2^{a}	10.0^{ab}	13.0^{a}	5.34^{c}	5.3^{c}	6.2^{bc}	2.89

^{abcd}Means within a row without a common superscript letter differ significantly at $P<0.05$, SEM=Standard error of means; NDIN: Neutral detergent insoluble nitrogen fiber; ADIN: Acid detergent insoluble nitrogen fiber

Gas and methane production

In vitro ruminal GP and CH_4 production at 24 and 48 h are shown in Table 2. Cellulase addition increased the overall mean GP ($P<0.05$) compared with zero addition either within 24 or 48 h (144 vs 126 ml/g DM - SEM 2.9 and 185 vs 178 ml/g DM-SEM 4.0 respectively at 24 and 48 h) while xylanase did not alter ($P>0.05$) the GP (123 vs 122 ml/g DM - SEM 2.6 at 24 h and 174 vs 174 mlg DM - SEM 4.1 at 48 h). Among the grasses, both enzymatic products treatments increased ($P<0.05$) the GP either at 24 or 48 h for Napier and at 24 h for Brachiaria while only the cellulase product added to Aruana enhanced the GP at all incubation times. No differences were detected for the other substrates. Incubation of Aruana and Napier in general resulted in lower ($P<0.001$) CH_4, followed by Brachiaria while sugar cane had the highest ($P<0.01$) value compared with all substrates both at 24 or 48 h incubation.

After 24 h of incubation, endoglucansae supplemented to Aruana and Napier increased ($P<0.05$) the CH_4. No effect was found ($P>0.05$) for the addition of the xylanase product. The enzymatic treatments did not affect the overall mean CH_4 in relation to TDOM or DNDF in any of the substrates. After 48-h of incubation, cellulase added to Buffel grass decreased ($P<0.05$) in relation to CH_4 DNDF while the opposite happened ($P<0.05$) with Aruana, Brachiaria and Napier. No effect was found for the other substrates. Xylanase addition failed to affect CH_4 in relation to TDOM among the grass substrates, but it decreased ($P<0.05$) CH_4 across all samples.

Table 2. Effects of the cellulase and xylanase products added to the different forage substrates on the gas (GP) and methane (CH_4) production.

Substrates	Enzyme[†]	Cellulase						Xylanase					
		GP (ml/g DM)		CH_4 (ml/g TDOM)		CH_4 (ml/g DNDF)		GP (ml/g DM)		CH_4 (ml/g TDOM)		CH_4 (ml/g DNDF)	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
All	Without	126.2[b]	178.1[b]	11.7	15.8	30.8	27.7	121.6	174.1	11.9	17.4[a]	42.0	24.6
	With	144.1[a]	184.8[a]	12.8	16.3	27.1	31.1	122.5	174.4	12.6	15.7[b]	32.7	20.7
	SEM	2.93	3.94	1.18	1.59	5.17	3.98	2.63	4.13	1.03	0.95	12.16	3.36
	P value												
Substrate (S)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Enzyme (E)		<0.001	0.018	0.187	0.717	0.320	0.233	0.627	0.924	0.342	0.014	0.279	0.106
S x E		<0.001	0.299	0.004	0.428	0.205	0.769	0.016	0.113	0.449	0.036	0.545	0.701
Aruana	Without	95[b]	144[b]	4.6[b]	9.7	8.5[b]	12.8	102	162	5.2	10.9	8.4	10.7
	With	103[a]	161[a]	6.4[a]	9.2	10.4[a]	13.1	101	157	5.6	9.9	9.2	9.7
	SEM	6.5	10.0	0.67	1.37	1.10	1.36	7.4	18.8	1.40	3.36	2.94	3.73
	P value	0.020	0.023	0.002	0.602	0.020	0.765	0.888	0.663	0.685	0.667	0.678	0.673
Brachiaria	Without	119[b]	175	9.8	12.5[b]	18.4	17.2[b]	116[b]	170	10.0	14.4	22.6	18.7
	With	142[a]	181	11.3	17.6[a]	17.2	25.0[a]	132[a]	170	11.0	13.2	17.9	16.4
	SEM	14.0	5.1	2.08	3.07	3.19	4.69	7.1	22.9	2.70	3.65	9.22	6.23
	P value	0.028	0.112	0.288	0.0264	0.5498	0.0262	0.005	0.971	0.574	0.611	0.438	0.581
Napier grass	Without	114[b]	158[b]	4.4[b]	5.0[b]	8.9	6.2[b]	114	140	5.1	7.0	8.9	12.6
	With	126[a]	168[a]	6.4[a]	7.1[a]	100.	12.1[a]	109	159	5.1	9.1	9.1	12.3
	SEM	7.9	6.2	1.32	1.48	1.98	1.69	11.7	24.5	1.49	2.84	3.69	4.00
	P value	0.038	0.027	0.039	0.048	0.407	0.0003	0.450	0.272	0.956	0.291	0.944	0.912
Sugarcane bagasse	Without	106	165	15.1	21.7	33.4	41.0	103	156	14.3	21.3	33.8	32.8
	With	111	159	11.6	21.5	41.6	44.3	99	145	13.3	15.8	28.4	22.2
	SEM	13.1	20.6	4.22	1.00	15.69	27.14	11.7	22.3	5.84	5.46	11.88	10.75
	P value	0.560	0.660	0.220	0.980	0.430	0.852	0.599	0.450	0.782	0.142	0.489	0.151
Sugarcane	Without	189	255	21.6	28.5	83.9	63.7	178	255	22.1	33.9	147.8	52.1
	With	191	259	20.5	26.3	87.1	51.5	176	255	21.9	30.0	101.6	43.5
	SEM	8.7	13.6	4.39	5.09	34.13	11.45	15.6	17.8	5.76	5.59	82.28	24.30
	P value	0.876	0.623	0.6917	0.523	0.885	0.309	0.8845	0.962	0.9621	0.299	0.3965	0.591
Buffel grass	Without	133	171	15.4	17.6	25.0[a]	25.5[a]	117	161	14.9	17.0	36.1	21.0
	With	127	180	13.1	15.7	20.7[b]	20.8[b]	118	160	18.9	16.3	24.4	20.3
	SEM	8.7	20.5	3.26	5.54	2.94	2.61	11.6	22.1	4.48	2.85	16.21	6.18
	P value	0.374	0.492	0.292	0.589	0.044	0.017	0.884	0.946	0.190	0.725	0.285	0.873

[†]Added at 7.5 or 0.46 unit (as µmol of sugar released/ml enzyme product/min)/500 mg DM of each substrate for endoglucanase and xylanase, respectively.

[ab]Within columns and substrates, means with different superscripts differ ($P<0.05$).

Table 3. Effects of the cellulase and xylanase products added to different grasses on the ruminal nutrient degradability (TDOM=truly degraded organic matter, DNDF=degraded neutral detergent fiber) and partitioning factor (PF).

Substrates	Enzyme[†]	Cellulase						Xylanase					
		TDOM (g/kg)		DNDF (g/kg)		PF (mg TDOM/mlGP)		TDOM (g/kg)		DNDF (g/kg)		PF (mg TDOM/ml GP)	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
All	Without	400[b]	493	187[b]	317	1.3	1.7[b]	397	520	203	354	1.4	1.8
	With	451[a]	499	274[a]	325	1.3	1.8[a]	397	529	201	371	1.4	1.9
	SEM	16.6	15.5	21.0	18.5	0.06	0.084	11.2	17.6	14.5	28.6	0.07	0.11
	P value												
Substrate (S)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Enzyme (E)		<0.001	0.587	<0.001	0.561	0.290	0.030	0.978	0.481	0.853	0.393	0.585	0.082
S x E		0.571	0.242	0.298	0.032	0.508	0.093	0.001	0.170	0.001	0.138	0.485	0.539
Aruana	Without	396	524	225[b]	403	1.7	2.5	410	556	276	444	1.7	2.1
	With	423	532	299[a]	414	1.7	2.1	391	583	253	477	1.6	2.4
	SEM	54.1	40.0	51.3	50.1	0.20	0.39	46.6	34.7	46.5	43.5	0.19	0.33
	P value	0.449	0.749	0.047	0.749	0.753	0.095	0.536	0.250	0.452	0.250	0.420	0.204
Brachiaria	Without	408[b]	547	219[b]	402	1.4	2.0[a]	378[b]	559	179[b]	418	1.4	1.9
	With	505[a]	526	347[a]	375	1.3	1.8[b]	448[a]	572	272[a]	435	1.4	2.0
	SEM	43.6	32.7	57.6	43.23	0.15	0.16	39.0	44.9	51.5	59.4	0.17	0.23
	P value	0.005	0.342	0.005	0.342	0.453	0.0718	0.018	0.659	0.018	0.659	0.564	0.644
Napier grass	Without	510	603	257[b]	388[b]	1.8	2.3	531	641	317	477	1.9	2.3
	With	574	625	380[a]	454[a]	1.8	2.2	531	602	317	421	1.9	2.3
	SEM	79.4	54.6	55.0	24.7	0.19	0.38	46.0	49.3	67.1	71.8	0.25	0.42
	P value	0.231	0.531	0.005	0.001	0.819	0.569	0.990	0.248	0.990	0.248	0.583	0.783
Sugarcane	Without	207	288	99	170	0.8	1.0	255	327	132	216	1.1	1.2
bagasse	With	244	268	119	148	0.9	1.0	191	323	108	212	0.9	1.3
	SEM	62.2	47.5	62.8	55.37	0.22	0.25	61.7	42.0	20.5	48.9	0.47	0.30
	P value	0.371	0.531	0.625	0.531	0.587	0.637	0.134	0.902	0.088	0.902	0.422	0.543
Sugarcane	Without	503	583	172	254	1.1	1.3	494	585	102	231	1.2	1.3
	With	540	568	148	201	1.2	1.2	485	658	71	367	1.2	1.5
	SEM	59.5	31.4	88.6	40.7	0.17	0.13	40.3	85.9	39.1	159.2	0.17	0.22
	P value	0.348	0.480	0.673	0.065	0.419	0.321	0.747	0.210	0.228	0.210	0.925	0.259
Buffel grass	Without	337	412[b]	174[b]	287[b]	1.0	1.5	312	452	184	336	1.2	1.8
	With	378	472[a]	245[a]	360[a]	1.2	1.7	335	434	218	314	1.2	1.8
	SEM	59.1	38.7	39.8	46.9	0.18	0.27	59.6	38.2	55.2	46.3	0.28	0.26
	P value	0.302	0.033	0.017	0.034	0.125	0.338	0.557	0.473	0.366	0.473	0.880	0.875

[†]Added at 7.5 or 0.46 unit (as µmol of sugar/ml of enzyme product/min)/500 mg DM of each substrate for endoglucanase and xylanase respectively

[ab]Within columns and substrates, means with different superscripts differ (P<0.05)

Ruminal degradability and partitioning factor

Cellulase addition increased ($P<0.05$) TDOM and DNDF in all grass species at 24 h but no effect was found after 48 h. Xylanase addition did not affect the degradability in any of the treatments (Table 3). Among the grasses, DNDF at 24 h was improved by the cellulase addition in Napier, Aruana and Buffel grass, both DNDF and TDOM were increased by cellulase and xylanase addition to Brachiaria after 24 h incubation (Table 3). For the substrates effect, Sugarcane had the highest ($P<0.05$) TDOM in all treatments while Aruana, Napier and Brachiaria presented the highest ($P<0.001$) DNDF values in the two incubation times compared with all substrates while Sugarcane bagasse showed the lowest ($P<0.001$) degradability both for TDOM and DNDF at 24 as well as at 48 h. The partitioning factor was increased ($P<0.05$) by both enzyme product at 48 h. For the cellulase product addition, the major improvement ($P<0.05$) in DNDF was found for Brachiaria, Napier and Aruana (Table 4) while for xylanase product, improvements in DNDF were highest in Brachiaria and Buffel grass ($P<0.05$) at 24 h compared with the other substrates. At 48 h, both of the enzymatic treatments resulted only in negligible TDOM and DNDF improvements (Table 4).

Table 4. Improvement on truly degraded organic matter (TDOM) and degraded neutral detergent fiber (DNDF) after incubation *in vitro* with the cellulase and xylanase products when added to different grasses (P value for the t-test)

Substrates	TDOM (g/kg)				DNDF			
	Cellulase†		Xylanase‡		Cellulase†		Xylanase‡	
	24h	*48h*	*24h*	*48h*	*24h*	*48h*	*24h*	*48h*
All	0.16	0.02	0.01	0.02	0.56	0.04	0.03	0.07
P value	<.001	0.406	0.943	0.372	<.001	0.335	0.957	0.186
Aruana	0.13	0.02	-0.04	0.05	0.32	0.10	-0.05	0.09
P value	0.001	0.695	0.250	0.295	0.001	0.235	0.584	0.285
Brachiaria	0.24	-0.03	0.18	0.03	0.62	-0.06	0.55	0.06
P value	0.007	0.116	0.001	0.627	0.014	0.124	0.001	0.568
Napier grass	0.12	0.01	0.04	-0.05	0.51	0.17	0.01	-0.10
P value	0.069	0.175	0.941	0.166	0.005	0.053	0.855	0.215
Sugarcane bagasse	0.20	-0.04	-0.15	0.01	0.29	-0.03	-0.35	0.02
P value	0.128	0.672	0.015	0.958	0.185	0.842	0.018	0.814
Sugarcane	0.09	-0.02	-0.01	0.12	0.17	-0.17	-0.31	0.42
P value	0.224	0.564	0.524	0.195	0.002	0.232	0.176	0.148
Buffel grass	0.16	0.15	0.13	-0.03	0.16	0.28	0.35	-0.04
P value	0.187	0.040	0.010	0.571	0.082	0.046	0.004	0.640

†‡Added to obtain 7.5 or 0.46 unit (as μmol of sugar/ml of enzyme product/min)/500 mg DM of each substrate for endoglucanase and xylanase, respectively).

Fermentation product characteristics

Both enzymatic treatments had no effect ($P>0.05$) on pH either after 24 or 48 h of incubation (6.8 ± 0.03 and 6.9 ± 0.02 respectively for cellulase and xylanase). The

cellulase product increased ($P<0.05$) ammonia concentrations in the incubation fluid only at 48 h compared with no enzyme addition (19 and 21 (SEM 1.1) mg/100 ml); xylanase had no affect ($P>0.05$) irrespectively to the time of incubation (15 ± 0.8 mg/100 ml at 24 h and 19 ± 0.4 mg/100 ml at 48 h).

None of the enzymatic treatments affected the acetate, valerate, isobutyrate, isovalerate, C2: C3 ratio and total SCFA concentrations in the incubation fluid at either incubation time 24 or 48 h. Overall, propionate and butyrate concentrations were increased ($P<0.05$) by the addition of the endoglucanase (Tables 5 and 6). Among the grasses, endoglucanase enhanced ($P<0.05$) acetate concentrations at 24 h with Aruana; propionate and butyrate with Napier grass and propionate with sugarcane. Xylanase decreased ($P<0.01$) the C2: C3 ratio with Brachiaria. Sugarcane with or without the enzymatic supplementations had the lowest C2: C3 ($P<0.05$) ratio either at 24 or 48 h and the highest total SCFA compared with the other substrates.

DISCUSSION

The increase ($P<0.05$) of the total overall mean of GP with the addition of the cellulase product indicates an increase in the degree of nutrient degradability of all substrates either at 24 or 48 h incubation. No such effect was observed by the use of the xylanase product. These results suggest that either the product is not effective in rumen fluid or that manufacturer's recommended dose of xylanase product was too low to enhance the *in vitro* rumen fermentation of the experimental forage substrates. This requires studies with higher doses to determine the optimal dose of xylanase for an effect on ruminal degradability as well as on CH_4 production. However, there is evidence that it would be possible to improve the effectiveness of enzyme preparations by increasing endoglucanase activity rather than xylanase activity (Beauchemin *et al.*, 2003). In turn, the cellulase product might have been even more effective when it would have been combined with a xylanase. It could even have been that cellulase product at the tested dose had a xylanase activity which was even higher than that of the dose of xylanase product investigated. This can be seen from the results of the activity test made where the cellulase product was found to provide 2.88 xylanase units and the actual xylanase product only 0.46 units. Thus, the increase in GP, DNDF and CH_4 at 24 h found with Aruana, Brachiaria and Napier grasses treated with the cellulase product may be partly related to a xylanase activity of the cellulase product since hemicellulose is the substrate most readily fermented by the ruminal microbes, followed by cellulose (Colombatto *et al.*, 2003). This finding is partly supported by the higher ($P<0.05$) hemicellulose content of Aruana and Brachiaria associated with lower fiber content since cellulase and xylanase usually act synergistically to hydrolyze forage cell wall more effective in cases of forages with lower fiber content (Vázquez *et al.*, 2011). These findings highlight the importance of matching the enzyme product to the forage substrate to achieve maximal benefit of using exogenous enzyme products in ruminant diets.

Table 5. Effect of the cellulase product added to the different grasses on the concentration (mmol/l) of short chain fatty acids (SCFA) in the incubation fluid.

Substrates	Enzyme†	Acetate		Propionate		Butyrate		Valerate		Isobutyrate		Isovalerate		C2: C3		Total SCFA	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
All	Without	31.5	31.3	7.6b	9.6	4.2b	4.8	0.82	0.89	0.15	0.25	0.85	1.07	4.3	3.3	45.1	47.9
	With	32.2	29.2	8.0a	9.6	4.3a	4.9	0.79	0.88	0.14	0.24	0.83	1.05	4.1	3.1	46.3	45.8
	SEM	3.05	2.00	0.27	0.31	0.11	0.12	0.076	0.031	0.024	0.011	0.046	0.031	0.41	0.18	3.21	2.30
	P value																
Substrate (S)		0.149	0.016	<0.001	<0.001	<0.001	<0.001	0.108	0.109	0.025	<0.001	0.001	0.001	0.001	0.001	0.061	0.001
Enzyme (E)		0.752	0.143	0.029	0.863	0.023	0.307	0.645	0.663	0.764	0.549	0.471	0.319	0.648	0.068	0.583	0.204
S x E		0.948	0.069	0.492	0.040	0.264	0.005	0.979	0.117	0.900	0.021	0.937	0.218	0.900	0.412	0.948	0.038
Aruana	Without	24.2b	31.7	5.4	8.2	3.5	4.2	0.75	0.82	0.17	0.26	0.88	1.10	4.4	3.9	34.9	46.2
	With	29.9a	28.1	6.4	8.2	4.0	4.4	0.81	0.82	0.19	0.26	0.94	1.09	4.7	3.4	42.2	42.9
	SEM	3.41	7.65	1.25	1.12	0.60	0.42	0.086	0.049	0.116	0.115	0.231	0.212	1.10	0.94	11.43	8.33
	P value	0.029	0.407	0.215	0.930	0.219	0.384	0.247	0.100	0.754	0.100	0.637	0.908	0.632	0.401	0.279	0.474
Brachiaria	Without	28.4	31.5	7.9	10.8	4.5	5.4	0.87	0.92	0.16	0.26	0.90	1.10	4.0	2.9	46.2	50.0
	With	31.8	24.6	8.4	9.7	4.5	4.9	0.78	0.85	0.15	0.21	0.87	1.00	3.4	2.5	43.1	41.3
	SEM	2.79	8.43	1.83	1.37	0.73	0.42	0.056	0.064	0.106	0.149	0.223	0.282	1.12	0.97	14.83	9.15
	P value	0.632	0.181	0.670	0.201	0.852	0.107	0.034	0.080	0.862	0.572	0.763	0.525	0.353	0.455	0.710	0.136
Napier grass	Without	32.8	28.1	6.9[b]	9.4	4.2[b]	5.1	0.86	0.97	0.18	0.31	0.90	1.17	4.7	3.4	45.9	49.5
	With	33.3	26.2	7.70[a]	9.1	4.8[a]	5.1	0.90	0.88	0.19	0.27	0.92	1.09	4.4	2.9	47.8	42.6
	SEM	5.76	2.69	0.43	0.69	0.30	0.67	0.104	0.097	0.114	0.166	0.268	0.318	0.87	1.15	5.88	13.28
	P value	0.876	0.236	0.031	0.358	0.021	0.904	0.462	0.134	0.872	0.662	0.550	0.622	0.421	0.402	0.574	0.370
Sugarcane	Without	25.0	25.7	6.6	8.3	3.8	4.1	0.83	0.85	0.19	0.23	0.94	1.06	3.8	3.1	37.3	40.3
bagasse	With	26.9	26.7	6.3	8.1	3.8	4.2	0.80	0.89	0.14	0.24	0.87	1.07	4.3	3.2	38.7	41.3
	SEM	8.94	12.00	0.86	1.22	0.39	0.67	0.15	0.17	0.09	0.17	0.19	0.45	1.07	0.99	9.91	14.63
	P value	0.700	0.881	0.531	0.798	0.708	0.798	0.686	0.687	0.377	0.887	0.466	0.967	0.404	0.748	0.801	0.903
Sugarcane	Without	32.1	35.5	10.6	12.5	4.8	5.5	0.83	0.88	0.08	0.20	0.69	0.96	3.0	2.8	49.1	55.5
	With	33.9	32.1	11.4	12.3	5.0	5.3	0.83	0.86	0.09	0.20	0.69	0.95	3.0	2.6	52.0	51.7
	SEM	3.57	10.56	0.52	1.18	0.19	0.94	0.180	0.161	0.031	0.145	0.061	0.309	0.40	0.63	3.45	12.96
	P value	0.367	0.563	0.039	0.716	0.125	0.720	0.972	0.761	0.565	0.966	0.671	0.936	0.816	0.490	0.180	0.590
Buffel grass	Without	33.1	30.7	7.1	8.4[b]	4.3	4.6	0.84	0.86	0.17	0.23	0.89	1.00	4.7	3.7	46.3	45.8
	With	32.7	37.6	7.2	9.9[a]	4.5	5.4	0.84	0.97	0.16	0.28	0.89	1.08	4.5	3.8	46.3	55.2
	SEM	1.60	10.24	0.93	1.07	0.44	0.84	0.10	0.21	0.13	0.16	0.22	0.39	2.25	0.71	16.47	12.31
	P value	0.959	0.260	0.732	0.038	0.384	0.142	0.949	0.364	0.922	0.574	0.532	0.733	0.889	0.802	0.995	0.208

†Added at 7.5 endoglucanase unit (as µmol of glucose/ml cellulase product/min)/500 mg DM of each substrate.

[ab]Within columns and substrates. means with different superscripts differ ($P<0.05$).

Table 6. Effect of the xylanase product added to the different grasses on the concentration (mmol/l) of short chain fatty acids (SCFA) in the incubation fluid.

Substrate	Enzyme[†]	Acetate		Propionate		Butyrate		Valerate		Isobutyrate		Isovalerate		C2: C3		Total SCFA	
		24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
All	Without	37,5	40,7	7,9	10,3	3,9	4,6	0,64	0,59	0,10	0,22	0,72	1,00	4,8	3,9	51,0	57,5
	With	38,9	40,0	9,8	10,2	4,0	4,5	0,62	0,60	0,11	0,21	0,73	0,99	4,6	4,0	54,2	56,7
	SEM	4,40	2,12	2,39	0,36	0,12	0,19	2.818	0,084	0,004	0,01	0,0176	0,032	0,63	0,18	5,08	2,58
	P value																
Substrate (S)		0,088	0,021	0,337	<,001	<,001	<,001	0,013	0,015	<,001	0,001	<,001	0,031	0,047	0,013	0,034	0,004
Enzyme (E)		0,653	0,671	0,271	0,552	0,774	0,765	0,225	0,994	0,288	0,378	0,964	0,524	0,652	0,725	0,363	0,640
S x E		0,692	0,926	0,453	0,582	0,814	0,914	0,537	0,999	0,405	0,414	0,366	0,514	0,308	0,756	0,785	0,912
Aruana	Without	34,2	39,4	6,5	9,8	3,6	4,5	0,65	0,70	0,11	0,24	0,77	1,04	5,3	4,1	45,8	55,5
	With	37,8	39,1	7,2	9,7	3,7	4,5	0,59	0,74	0,12	0,25	0,72	1,10	5,3	4,1	50,1	55,4
	SEM	5,15	4,68	0,78	1,49	0,51	0,18	0,588	0,715	0,088	0,147	0,118	0,156	0,49	0,60	5,02	5,26
	P value	0,243	0,927	0,147	0,996	0,657	0,547	0,834	0,884	0,782	0,835	0,428	0,492	0,891	0,951	0,165	0,982
Brachiaria	Without	34,8	36,6	8,0	10,6	4,3	5,0	0,70	0,73	0,10	0,20	0,76	0,97	4,4	3,5	48,7	54,2
	With	33,2	39,4	8,6	11,1	4,3	5,2	0,68	0,72	0,10	0,21	0,79	0,98	3,9	3,6	47,7	57,7
	SEM	4,35	2,82	0,81	1,25	0,83	0,23	0,664	0,434	0,072	0,108	0,068	0,209	0,18	0,40	5,28	3,48
	P value	0,519	0,124	0,242	0,459	0,976	0,141	0,970	0,954	0,932	0,821	0,486	0,906	0,006	0,682	0,735	0,117
Napier grass	Without	34,0	40,5	7,8	10,3	3,9	4,7	0,64	0,71	0,12	0,25	0,8	1,0	4,3	3,9	47,3	57,6
	With	45,0	39,6	7,5	9,5	3,9	4,5	0,66	0,70	0,12	0,22	0,8	1,1	6,1	4,2	58,0	55,8
	SEM	19,44	6,27	0,74	1,64	0,60	0,39	0,601	0,443	0,084	0,132	0,09	0,13	3,07	0,22	1,69	7,81
	P value	0,330	0,843	0,433	0,389	0,991	0,235	0,943	0,966	0,884	0,649	0,553	0,853	0,319	0,093	0,346	0,675
Sugarcane	Without	37,6	38,4	6,8	9,3	3,4	3,9	0,58	0,42	0,10	0,22	0,75	1,00	5,7	4,0	49,2	53,3
bagasse	With	35,1	36,5	16,6	8,6	3,5	3,7	0,60	0,43	0,12	0,20	0,76	0,94	3,8	4,3	56,6	50,4
	SEM	9,85	18,03	19,59	2,84	0,54	1,07	0,617	0,539	0,076	0,161	0,077	0,315	3,52	1,25	23,37	21,75
	P value	0,644	0,847	0,379	0,656	0,865	0,732	0,952	0,981	0,749	0,850	0,937	0,728	0,347	0,657	0,565	0,809
Sugarcane	Without	47,4	47,5	11,4	12,5	4,8	5,1	0,68	0,52	0,07	0,20	0,60	0,99	4,2	3,8	64,9	66,8
	With	50,2	45,3	11,9	12,8	5,0	5,1	0,65	0,49	0,08	0,18	0,61	0,93	4,1	3,6	68,5	64,8
	SEM	26,33	6,74	2,49	2,28	0,76	0,95	0,608	0,634	0,055	0,167	0,087	0,246	1,70	0,34	28,73	9,92
	P value	0,847	0,556	0,670	0,796	0,559	0,984	0,919	0,945	0,826	0,883	0,711	0,657	0,945	0,182	0,817	0,712
Buffel grass	Without	37,5	41,8	7,5	9,8	3,9	4,4	0,63	0,50	0,11	0,22	0,72	1,01	5,0	4,3	50,3	57,7
	With	32,3	40,2	7,2	9,6	3,7	4,4	0,57	0,50	0,10	0,21	0,70	0,97	4,5	4,2	44,5	55,9
	SEM	7,15	10,80	1,04	2,91	0,31	1,09	0,546	0,668	0,085	0,153	0,083	0,237	0,48	0,42	0,45	14,93
	P value	0,228	0,795	0,552	0,886	0,247	0,973	0,848	0,985	0,886	0,968	0,663	0,738	0,129	0,746	0,230	0,823

[†]Added at 0.46 xylanse unit (as µmol of xylose/ml of xylanase product/min)/500 mg DM of each substrate.

[ab]Within columns and substrates. means with different superscripts differ ($P<0.05$).

Rather than by the enzyme experimental dose, it seems that the effect of the experimental enzymes on CH_4 production was influenced by the fiber composition and thus the forage quality. The chemical composition suggest that both Aruana and Napier are good quality grasses because they contain more ($P<0.05$) protein and less fiber compared with sugarcane bagasse, sugarcane and Buffel grass. In CH_4, Aruana and Napier responded to cellulase treatment while no differences were detected for the lower quality forages. This prevented an overall cellulase effect on CH_4 emission. Considering the simultaneous improvements in GP, rumen degradability and SCFA enhancement, the good quality forage with or without enzyme treatment still would probably result in less CH_4 emissions when related to intake of feed or digestible nutrients. Additionally, low digestibility forages are digested slowly and remain in the rumen for longer periods, limiting rumen capacity, decreasing feed intake and providing more time to synthesize CH_4 emission (Hart *et al.*, 2009). Finally, the expected CH_4 emission by product unit (milk yield or weight gain per animal) values would be lower where gains or yields per animal are greater (Goel and Makkar, 2012). Holtshausen *et al.* (2011) reported that the use of fibrolytic enzymes improved the energy availability of the diet and, consequently developmental enzyme product improved fat corrected milk production efficiency by 11.3% in early-lactation dairy cows with their inherently low feed intake in relation to requirements.

The cluster of variables that were positively related in the high quality forages pasture quality to the CH_4 reduction while enhancing the rumen fermentation process is in agreement with previous studies (Goel and Makkar, 2012; Abdalla *et al.*, 2012a, b; Soltan *et al.*, 2012). These included the general improvements in the GP, NDF degradability, NH_3-N and SCFA concentrations associated with the CH_4 reduction found for Aruana and Napier compared with the other substrates. These results suggest that the use of the high quality grasses improved the efficiency of microbial protein synthesis by increase the incorporation of ruminally degraded OM into microbial cells, while the percentage partitioned into CH_4 was decreased (Blümmel *et al.*, 1997). This assumption is partly supported by the increasing ($P<0.05$) in branched SCFA (iso C4 and iso C5) concentrations, which are known to be microbial growth promoting, especially in fiber degrading bacteria (Blümmel *et al.*, 1997) and also by the high PF values (an indicator for microbial protein synthesis) found for both Aruana and Napier associated with the CH_4 reduction compared with sugarcane bagasse as a representative of low quality forage in all treatments and incubation times.

Abdalla *et al.* (2012a); Goel and Makkar (2012) suggested that CH_4 production was associated with the increase in fermented and digested OM. These seem to have to be the case with sugarcane, because the CH_4, GP, total and individual SCFA were highest compared with all treatments either in 24 or 48 h. A probable reason for such a response has been proposed to be the soluble carbohydrates (saccharose) present in sugarcane which would provide energy that would lead to rapid microbial growth and thus, shorten the lag time for fibrolytic microbial colonization (Elwakeel *et al.*, 2007).

In light of those findings, it seems as if high sugar grass may stimulate the fermentation process but by this way also results in high amounts of CH_4 per unit of degraded OM.

Morgavi *et al.* (2001) reported that exogenous enzymes fed to animals are stable in the rumen against ruminal proteases for a period of 6 h. Elwakeel *et al.* (2007) pointed out the importance of adsorption and binding of the enzyme to substrate before feeding to allow proper attachment and protection against degradation by rumen proteases. Thus, in the present study, a pre-treatment of 18 h of the forage substrates with the enzyme products before incubation with ruminal fluid was applied to enhance the beneficial effects of the enzymes on ruminal fermentation by the creation of a stable enzyme-feed complex. This pre-treatment may even have alterated the fiber structure, which would further stimulate microbial colonization (Colombatto *et al.*, 2003; Giraldo *et al.*, 2008). In the present study, cellulase overall yielded more positive responses in TDOM, DNDF and fermentation end product synthesis at 24 h than at 48 h. This illustrates that the addition of the cellulase promoted a fast rate of feed degradation in the rumen and microbial cellulases caught up when the incubation time increased (Colombatto *et al.*, 2003; Elwakeel *et al.*, 2007).

The still observed general increase in the GP, TDOM, DNDF and NH_3-N associated with higher PF by cellulase treatment at 48 h probably originated from enzyme applied as well as the increase in the bacterial population (Morgavi *et al.*, 2001). Wang *et al.* (2001) found that the addition of enzymes increased the number of cellulolytic bacteria almost by 10-fold. Reducing sugars released from the digested portion of the feed were found to attract bacteria to the site of digestion and enhance the attachment of bacteria to undigested feed particles (Wang *et al.*, 2001).

Even though there were no differences detected in the effect of the cellulase product treatment on the overall mean of SCFA concentration, there was a significant interaction found between the enzyme and forage substrate. This might be due to the observation that the response of the high quality forage substrates to the enzymatic treatment was greater than that of the low quality grasses. Zhou *et al.* (2011) concluded that exogenous fibrolytic enzymes do not dramatically influence the total population and species composition of the ruminal methanogenic community. Thus, the greater CH_4 produced by incubating the cellulase-treated Aruana and Napier grass was consistent with the change in SCFA pattern and suggest that the CH_4 increase was a reflection of the improvement of fermentation and fiber degradation and not of a modified for methanogenic community. The enhancement of acetate and butyrate concentrations by incubating these forages confirmed this assumption since acetate and butyrate production are associated with the release of H_2 which can be used by methanogens to form CH_4. In turn, a shift in SCFA production towards more propionate without changes in the total SCFA and pH is usually expected with an inhibition of CH_4 production (Goel and Makkar, 2012). This was the case for Buffel grass substrate, the forage with the highest fiber content, when treated with the cellulase product compared

with no addition. However, no such effect was found for sugarcane bagasse. Thus the improvement in fiber degradability in forages with high cellulose content by endoglucanase treatment produce less CH_4 and higher propionate, unless it is highly lignified sugarcane bagasse which may negatively affect the general rumen fermentation processes and nutrient degradability even with the endoglucanase treatment. Still, this assumed association between CH_4 production and forage cellulose and lignin content needs to be further investigated using a larger set of plant samples and variables measured.

CONCLUSION

The present results show that responses to the manufacturer's recommended dose of the exogenous fibrolytic enzymes products added to forages can vary substantially for different grass types. The responses were best to the cellulase and this with Aruana and Napier. In order to optimize the efficiency of application of fibrolytic enzymes also with other grasses further doses need to be evaluated and this under *in vivo* conditions as well.

ACKNOWLEDGEMENT

The authors wish to thank CNPq (Brazil) and International Atomic Energy Agency (IAEA, Austria) for financial support and Prof. Michael Kreuzer (ETH Zurich) for comments and English corrections.

REFERENCES

Abdalla, A.L., Louvandini, H., Sallam, S.M.A.H., Bueno, I.C.S., Tsai, S.M. and Figueira A.V.O. 2012a. *In vitro* evaluation, *in vivo* quantification, and microbial diversity studies of nutritional strategies for reducing enteric methane production. *Tropical Animal Health and Production*, 44: 953-964.

Abdalla, A.L., Soltan, Y.A., Silva, L.R.F., Louvandini, H., Mcmanus, C.M., Lucas, R.C., Morsy, A.S., Beleosoff, B.S. and Abdalla Filho, A.L. 2012b. Good quality grass pasture decreases rumen methane production *in vitro*. Proceedings of the ASA, CSSA, and SSSA International Annual Meetings, 21-24 Oct., 2012, Cincinnati, USA. (Abstr. 257-42).

AOAC. 1995. *Official Methods of Analysis*, 16th ed. Arlington, VA.

Bailey, M.J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*, 23: 257-270.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science,* 81: 37-47.

Blümmel, M., Makkar, H.P.S. and Becker, K. 1997. *In vitro* gas production: a technique revisited. *Journal of Animal Physiology and Animal Nutrition*, 77: 24-34.

Bueno, I.C.S., Cabral Filho, S.L.S., Gobbo, S.P., Louvandini, H., Vitti, D.M.S.S. and Abdalla, A.L. 2005. Influence of inoculum source in a gas production method. *Animal Feed Science and Technology,* 123: 95-105.

Colombatto, D., Mould, F.L., Bhat, M.K., Morgavi, D.P., Beauchemin, K.A. and Owen, E. 2003 Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal micro-organisms *in vitro*. *Journal of Animal Science,* 81: 1040-1050.

Colombatto, D. and Beauchemin, K.A. 2003. A proposed methodology to standardize the determination of enzymic activities present in enzyme additives used in ruminant diets. *Canadian Journal of Animal Science,* 83: 559-568.

Eckard, R.J., Grainger, C. and De Klein, C.A.M. 2010. Options for the abatement of methane and nitrous oxide from ruminant production. *Livestock Science*, 130: 47-56.

Elwakeel, E.A., Titgemeyer, E.C., Johnson, B.J., Armendariz, C.K. and Shirley, J.E., 2007. Fibrolytic enzymes to increase the nutritive value of dairy feedstuffs. *Journal of Dairy Science,* 90: 5226-5236.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J., Ramos, S. and Carro, M.D. 2008. Influence of direct- fed fibrolityc enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. *Journal of Animal Science,* 86: 1617-1623.

Goel, G. and Makkar, H.P.S. 2012. Methane mitigation from ruminants using tannins and saponins, a status review. *Tropical Animal Health and Production*, 44: 729-739.

Hart, K.J., Martin, P.G., Foley, P.A., Kenny, D.A. and Boland, T.M. 2009. Effect of sward dry matter digestibility on methane production, ruminal fermentation, and microbial populations of zero-grazed beef cattle. *Journal of Animal Science,* 87: 3342-3350.

Holtshausen, L., Chung, Y.H., Gerardo-Cuervo, H., Oba, M. and Beauchemin, K.A. 2011. Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. *Journal of Dairy Science,* 94: 899-907.

Longo, C., Bueno, I.C.S., Nozella, E.F., Goddoy, P.B., Cabral Filho, S.L.S. and Abdalla, A.L. 2006. The influence of head-space and inoculum dilution on *in vitro* ruminal methane measurements. *International Congress Series*, 1293: 62-65.

Mertens, D.R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feed with refluxing beakers or crucibles: collaborative study. *Journal of AOAC International*, 85: 1217-1240.

Morgavi, D.P., Beauchemin, K.A., Nsereko, V.L., Rode, L.M., McAllister, T.A., Iwaasa, A.D., Wang, Y. and Yang, W.Z. 2001. Resistance of feed enzymes to proteolytic inactivation by rumen micro-organisms and gastrointestinal proteases. *Journal of Animal Science,* 79: 1621-1630.

Palmquist, D. and Conrad, H. 1971. Origin of plasma fatty acids in lactating cows fed high grain or high fat diets. *Journal of Dairy Science,* 54: 1025-1031.

Preston, T.R. 1995. Biological and chemical analytical methods. In: *Tropical Animal Feeding: A Manual for Research Workers* (Ed. T.R. Preston), FAO, Rome, pp. 181-239.

SAS. 2002. *Version 9.1.3.* SAS Institute Inc., Cary, NC.

Soltan, Y.A., Morsy, A.S., Sallam, S.M.A., Louvandini, H. and Abdalla, A.L. 2012. Comparative *in vitro* evaluation of forage legumes (prosopis, acacia, atriplex, and leucaena) on ruminal fermentation and methanogenesis. *Journal of Animal and Feed Sciences*, 21: 759-772.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., Mcallan, A.B. and France, J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology,* 48: 185-197.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3597.

Vázquez, A.G., Martínez, G.M. and Rodríguez, J.P. 2011. Comparison of *in vitro* degradation of elephant grass and sugarcane by exogenous fibrolytic enzymes. *African Journal of Microbiology Research*, 5: 3051-3053.

Wang, Y., McAllister, T.A., Rode, L.M., Beauchemin, K.A., Morgavi, D.P., Nsereko, V.L., Iwaasa, A D. and Yang, W.Z. 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the rumen simulation technique (Rusitec). *British Journal of Nutrition*, 85: 325-332.

Wood, T.M. and Bhat, M.K. 1988. Methods for measuring cellulase activities. *Methods in Enzymology*, 160: 87-112.

Zhou, M., Chung, Y.H., Beauchemin, K.A., Holtshausen, L., Oba, M., McAllister, T.A. and Guan, L.L. 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. *Journal of Applied Microbiology*, 111: 1148-1158.

Animal Nutrition and Feed Technology (2013) 13: 569-574

Influence of Exogenous Enzymes on *In Vitro* Ruminal Degradation of Ensiled Rice Straw with DDGS#

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ABSTRACT

Gado, H.M., Salem, A.Z.M., Camacho, L.M., Elghandour, M.M.Y. and Salazar, M.C. 2013. Influence of exogenous enzymes of ZAD on in vitro ruminal degradation of ensiled rice straw with DDGS. Animal Nutrition and Feed Technology, 13: 569-574.

The objective of this study was to determine the effect of exogenous enzymes (ENZ) on nutrient profile and ruminal degradability of rice straw (RS), distillers dried grains with solubles (DDGS) and their mixture (RS with 10% DDGS). Ten samples of each fibrous feed were mixed with ZAD® (mixture of cellulases, xylanaxes, proteases and alpha amylase). ENZ was added at 0, 1 and 3 L to one ton of the fibrous feeds and the mixture was ensiled for 30 days. Feed samples were incubated for 72 h in rumen liquor of sheep to determine the degradability of DM, NDF and ADF. Pretreatment of feeds and their mixture (RS and DDGS) with ENZ at 3 L were increased ($P<0.01$) the degradation of NDF and ADF. Degradation fractions (a, b, (a+b) and c) of feeds were improved ($P<0.01$) at 3 L of ENZ, except the *c* of NDF and ADF of RS which were not affected by ENZ treatment. The results suggested a strong potential in improving digestion of RS and DDGS as well as their mixture with the pretreatment with ENZ. The dose of 3 L/ton of fibrous product improved the DM, NDF and ADF degradability.

Key words: DDGS, Degradability, Exogenous enzymes, Fiber fractions, Rice straw.

INTRODUCTION

Fibre degradation in the rumen is not fully efficient because the fibre fraction recovered from faeces is fermentable (Krause *et al.,* 2003). In recent years, abundant researches focused on addition of exogenous enzyme on fibre digestibility of *in vitro* rumen fermentation (Hristov *et al.,* 2008; Salem *et al.,* 2012), however, as opinions vary, no defined conclusion can be drawn about the effect of exogenous enzymes products on fibre digestibility. The enzymes preparation ZAD® is biotechnical product made from anaerobic bacteria which convert the polysaccharide into monosaccharide by specific enzymes. Gado *et al*. (2011) reported that ZAD® improved nutrients digestibility, live body weight gain and feed conversion of wheat straw in sheep. The

#This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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ZAD® is mixture of enzymes from anaerobic bacteria that had a beneficial effect on digestibility of low quality roughages. DDGS are highly valued as an animal feed for its nutrient content (Kingsly *et al.,* 2010). High protein and high energy content make distillers DDGS a unique ingredient for ruminant diets, but variation in composition reduces nutritional quality and market value (Belyea *et al.,* 2010). The objectives of the present study aimed at investigation of the effect of an anaerobic enzyme (ENZ) on nutrient profile and ruminal degradability of rice straw, DDGS and their mixture (RS with 10% DDGS).

MATERIALS AND METHODS

Ten samples of one kg for each of fibrous feeds sample (RS, DDGS) or their mixture (RS with 10% DDGS), on a DM basis, were collected from different sites of each fibrous material bulk and mixed with an enzyme product ZAD® (ENZ, according to the manufacturer; Bactizad Inc., Cairo, Egypt). The ZAD® is a product in a powder form manufactured by the Academy of Scientific Research and Technology, Egypt, and contains enzymes of cellulases (7.1 unit g^{-1}), xylanases (2.3 unit g^{-1}), α-amylase (61.5 unit g^{-1}) and protease (29.2 unit g^{-1}) obtained through an anaerobic fermentation process of anaerobic bacteria. Enzymes activities of ZAD® were determined according to the methods mentioned in Salem *et al.* (2013). Chemical composition of fibrous feeds are presented in Table 1.

Table 1. Chemical composition (%±SD) of rice straw, DDGS and their mixture [*n=10*]

	Rice straw (RS)	DDGS	RS with 10% DDGS
DM	90.0±2.31	90.0±1.23	96.1±2.11
CP	3.8±0.98	26.8±1.22	6.0±0.82
NDF	65.5±2.13	43.6±1.34	50.1±2.40
ADF	48.0±1.37	10.7±0.43	46.7±1.56

Samples of each fibrous material (*i.e.,* RS, DDGS and RS with DDGS) were chopped at 5 cm and mixed with ENZ at three levels (0, 1 or 3 L/ton of fibrous material) and moistened to a relative humidity of approximately 50%. Sugarcane molasses was added at 10 kg/ton of DM fibrous material. Enzymes of ZAD® was sprayed inside the mixture in a unify way to all of the contents without changing the humidity contents between treatments. After the mixture, the whole contents were transferred to a baling machine to press the whole contents together that followed by a plastic raving machine to isolate the whole contents from air (anaerobic condition) and ensiled for 30 days. Samples of ensiled RS and DDGS or their mixture were ground through a 1 mm screen (Wiley mill, Arthur H. Co., Philadelphia, PA, USA) for chemical analysis (Table 1). Samples were analysed for DM (934.01), ash (942.05), N (954.01) and EE (920.39) as per AOAC (1997). The NDF (Van Soest *et al.*, 1991), ADF and lignin (AOAC 1997; 973.18) were analysed using an ANKOM 200 Fibre Analyzer Unit (ANKOM Technology Corporation, Macedon, NY, USA). NDF was assayed with heat-stable alpha-amylase and with sodium sulfite in the NDF. Both NDF and ADF were expressed without residual ash.

The *in vitro* degradation of samples was carried out according to the method of Tilley and Terry (1963) in ruminal fluid collected from three sheep of 45 kg live weight and feed on total mixed ration of concentrate and roughages (1.1). Sheep were fitted with permanent rumen fistula. Ruminal fluid was collected before the morning feeding and filtrated through four layers of cheesecloth and filled with O_2-free CO_2 headspace. Samples (1 g) were weighed in polypropylene tubes with runner stopper. Forty ml of saliva were added to samples with and 10 ml of filtrated ruminal liqueur. Tubes were then incubated in water bath at 39°C for 2, 4, 6, 8, 12, 24, 48, 72 h. after incubation, tubs were filtrated and undigested residuals were recovered and dried at 65°C for 24 h and weighed. Subsample of the undigested residual was used for NDF and ADF contents determination. Incubations were done in different runs and days.

It used five tubs for each treatment during the *in vitro* incubation. Kinetics of *in vitro* degradation was measured by fitting the data in the Gompertz model (Susmel *et al.,* 1999) as follows:

$$dis(t) = (a+b) * \exp[(-c)\exp(-Dt)]$$

Where, dis(t) is the degradation of sample (g/kg) at time 't'; 'a' is the rapid soluble fraction (g/kg) at t=time (h); 'b' is the insoluble, but potentially degradable fraction (g/kg); 'c' is the degradation rate of a+b; 'D' is a parameter to measure the degradation. According to Gompertz model, the fractional rate of degradation varies as a function of time, and the average value (*i.e.*, a constant comparable to the exponential rate of degradation).

Data of DM, NDF and ADF degradability at each incubation time were fitted in the "NLIN" procedure of SAS (1999) for calculation the degradation fraction of a, b and c. Data of *in vitro* degradation wasanalysed as complete randomized design using the GLM procedure of SAS (1999) considering the individual samples within each fibrous material as experimental unit. Linear and quadratic orthogonal contrasts within each fibrous species were analysed.

RESULTS

The RS showed the highest ($P<0.05$) NDF and ADF contents compared to the DDGS while the CP content was the highest in DDGS. Application of ENZ at 3 L increased the quadratically ($P<0.01$) the degradation of NDF and ADF of RS, DDGS and their mixture, and was followed by treatment of 1 L of ENZ versus control treatment (Table 2). Increasing the level of ENZ from 0-3 L before ensiling RS, DDGS and their mixture improved the quadratically ($P<0.01$) degradation fractions (a, b, (a+b) and c) of nutrients but had no effect on the c of NDF and ADF of RS. In general, the ENZ increased the potentially degradable fractions of NDF and ADF of DDGS by 11%, the potentially degradation fraction of NDF and ADF of RS plus DDGS by 6 and 8%, respectively and the degradation rate of NDF and ADF of RS by 24% (Table 3).

Table 2. Effect of level (0, 1 and 3 L ton^{-1} of the fibrous feed) of ENZ on fiber fraction (%) of ensiled RS, DDGS and their mixture for sheep

		ENZ (L ton^{-1})			SEM	Contrast†	
		0	1	3		L	Q
RS	NDF	65.5	60.1	50.2	4.45	0.009	0.024
	ADF	48.0	41.2	32.6	3.26	0.007	0.035
	ADL	9.0	8.7	8.6	0.78	0.181	0.062
DDGS	NDF	43.7	39.8	31.2	5.30	0.009	0.034
	ADF	18.7	13.4	10.1	2.89	0.008	0.016
	ADL	3.0	2.8	2.7	0.65	0.151	0.205
RS with 10% DDGS	NDF	63.6	58.4	49.3	4.82	0.008	0.016
	ADF	46.2	40.0	30.1	4.53	0.006	0.034
	ADL	8.0	7.9	7.8	0.34	0.191	0.042

†Probability of a linear (L) or quadratic (Q) effect of ENZ level.

Table 3. Effect of ENZ levels on *in vitro* degradation† of DM and fibre fractions of RS, DDGS and their mixture in sheep

		ENZ, L ton^{-1}			SEM‡	Contrast§	
		0	1	3		L	Q
RS	DM						
	a (%)	11.0	17.3	22.6	4.66	0.006	0.024
	b (%)	33.4	39.2	44.7	3.12	0.009	0.035
	a+b (%)	44.4	56.5	67.3	2.88	0.006	0.041
	c (%/h)	2.3	3.4	4.6	0.68	0.008	0.021
	NDF						
	b (%)	41.5	44.6	48.6	1.45	0.009	0.016
	c (%/h)	2.1	2.4	2.7	0.42	0.240	0.340
	ADF						
	b (%)	38.2	41.1	44.7	1.44	0.008	0.019
	c (%/h)	2.4	2.6	2.8	0.61	0.190	0.048
DDGS	DM						
	a (%)	28.7	29.4	31.4	1.44	0.005	0.036
	b (%)	46.1	49.8	53.4	2.62	0.009	0.042
	a+b (%)	74.8	79.2	84.8	1.89	0.007	0.026
	c (%/h)	3.8	4.0	4.9	0.79	0.007	0.042
	NDF						
	b (%)	45.4	47.4	51.5	1.93	0.008	0.035
	c (%/h)	3.2	3.5	3.9	0.53	0.008	0.012
	ADF						
	b (%)	49.9	50.6	55.6	1.99	0.009	0.043
	c (%/h)	3.4	3.5	3.9	0.29	0.006	0.051
RS with 10% DDGS	DM						
	a (%)	15.6	26.4	33.8	3.73	0.009	0.043
	b (%)	34.2	46.3	55.1	2.47	0.008	0.031
	a+b (%)	49.8	72.7	88.9	4.83	0.008	0.026
	c (%/h)	3.2	4.3	5.4	0.29	0.006	0.04
	NDF						
	b (%)	42.5	48.6	53.6	3.81	0.007	0.042
	c (%/h)	2.8	3.1	3.6	0.33	0.006	0.043
	ADF						
	b (%)	41.8	42.2	45.8	1.69	0.008	0.036
	c (%/h)	2.6	3.1	3.4	0.29	0.006	0.046

†a, soluble fraction; b, potentially degradable fraction; a+b, total degradation; c, degradation rate.

‡SEM, standard error of the mean.

§Probability of a linear (L) or quadratic (Q) effect of ENZ level.

DISCUSSION

Enzyme level and fiber degradability

The DM degradation of RS was improved by enzymes; however, ENZ increased both potentially degradable fraction and degradation rate of NDF and ADF. The positive results of enzymes on *in vitro* degradation of rice straw fibre are consistent with those reported by Yang *et al*. (1999). However, the mode of action by which ENZ improves degradation has not been elucidated, although it has been suggested that it could be related to the fact that ENZ may enhance rumen enzyme activity (Hristov *et al.,* 2008) due to increments of soluble carbohydrates released from undigested feed particles, which provides additional energy for microbial growth and shortening the lag time for microbial colonization (Sutton *et al.,* 2002).

Additionally, it could contribute to the increase of the soluble fraction that would be expected if feeds are directly treated with enzymes, because this pretreatment has been shown to start fiber degradation and to reduce the NDF content of different feeds (Giraldo *et al.,* 2008). The release of sugars from feeds arises at least partially from the solubilization of NDF and ADF (Hristov *et al.,* 2008). This is consistent with increased soluble fraction and rate of *in situ* digestion (Hristov *et al.,* 2008). The DDGS pretreatment with ENZ could assist in elevating NDF and ADF degradability as it appeared in the obtained results.

Enzymes and type of fiber

Our results showed that the ENZ were effective on RS and DDGS and their mixture plus ENZ would be expected to increase fiber digestion by increasing the rate of ruminal digestion of the potentially digestible NDF fraction (Yang *et al.,* 1999). The large effects of the ENZ preparation on RS could be due to their enzymatic activities and levels. The ENZ in our study at 3 L, have improved the degradation of RS followed by DDGS. The mixture of RS and DDGS showed a positive associative effect on the increase of soluble part of their fiber. Salem *et al*. (2012) reported that sun-drying of roughage and addition of ENZ had a beneficial impact on fibre digestibility. Different type of ENZ affected the highest specific growth rates of bacteria or yeast (Lamsal *et al.,* 2012).

The ruminal insoluble potentially degradable fraction (b) of grass hay DM and its fractional rate of degradation (c) were increased ($P<0.05$) by ENZ treatment. Supplementation with ENZ also increased ($P<0.01$) effective and potential degradability of grass hay DM and NDF (Giraldo *et al.,* 2008). This result was consistent with nutrients digestion were higher ($P<0.05$) in ensiled orange pulp with ENZ and digestible DM was increased by 18%, whereas the fiber fractions (NDF and ADF) were increased by 93 and 47% with similar ensiled orange pulp with ENZ (Gado *et al.,* 2011).

CONCLUSIONS

The use of exogenous enzymes showed strong potential in improving the degradation of fibrous materials such as RS and DDGS. A dose of 3 L of ENZ/ton of fibrous feed product improved the DM, NDF and ADF degradation of RS, DDGS and their mixture. The increase in soluble fraction, potentially degradable fraction, total degradation and degradation rate were significantly higher at 3 L of ENZ treatment than 1 L with respect to the source of fiber.

REFERENCES

AOAC. 1997. *Official Methods of Analysis*, 16th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Belyea, R.L., Rausch, K.D., Clevenger, T.E., Singh, V., Johnston, D.B. and Tumbleson, M.E. 2010. Sources of variation in composition of DDGS. *Animal Feed Science and Technology,* 159: 122-130.

Gado, H.M., Salem, A.Z.M., Odongo, N.E. and Borhami, B.E. 2011. Influence of exogenous enzymes ensiled with orange pulp on digestion and growth performance in lambs. *Animal Feed Science and Technology,* 165: 131-136.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2008. Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage: concentrate ratios. *Animal Feed Science and Technology,* 141: 306-325.

Hristov, A.N., Hristov, A.N., Basel, C.E., Melgar, A., Foley, A.E., Ropp, J.K., Hunt, C.W. and Tricarico, J.M. 2008. Effect of exogenous polysaccharide-degrading enzyme preparations on ruminal fermentation and digestibility of nutrients in dairy cows. *Animal Feed Science and Technology,* 145: 182-193.

Kingsly, A.R.P., Ileleji, K.E., Clementson, C.L., Garcia, A., Maier, D.E., Stroshine, R.L. and Radcliff, S. 2010. The effect of process variables during drying on the physical and chemical characteristics of corn dried distillers grains with solubles (DDGS) - Plant scale experiments. *Bioresource Technology*, 101: 193-199.

Krause, D.O., Denman, S.E., Mackie, R.I., Morrison, M., Rae, A.L., Attwood, G.T. and McSweeney, C.S. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiology Reviews*, 27: 663-693.

Lamsal, B.P., Pathirapong, P. and Rakshit, S. 2012. Microbial growth and modification of corn distillers dried grains with solubles during fermentation. *Industrial Crops and Products*, 37: 553-559.

Salem, A.Z.M., Gado, H.M., Colombatto, D. and Eghandour, M.M.Y. 2013. Effect of exogenous enzymes on nutrient digestibility, ruminal fermentation and growth performance in beef steers. *Livestock Sciences*, 154: 69-73.

Salem, A.Z.M., Hassan, A.A., Khalil, M.S., Gado, H.M., Alsersy, H. and Simbaya, J. 2012. Effects of sun-drying and exogenous enzymes on nutrients intake, digestibility and nitrogen utilization in sheep fed *Atriplexhalimus* foliages. *Animal Feed Science and Technology*, 171: 128-135.

SAS. 1999. *User's Guide: Statistics.* Version 8. SAS Institute Inc., Cary, NC, USA.

Susmel, P., Spanghero, M. and Stefanon, B. 1999. Interpretation of rumen degradability of concentrate feeds with a Gompertz model. *Animal Feed Science and Technology*, 79: 223-237.

Sutton, J.D., Phipps, R.H., Deaville, E.R., Jones, A.K. and Humphries, D.J. 2002. Whole-crop wheat for dairy cows: Effects of crop maturity, a silage inoculant and enzyme added before feeding on food intake and digestibility and milk production. *Journal of Animal Science*, 74: 307-318.

Tilley, J.M. and Terry, R.A. 1963. A two-stage technique for the *in vitro* degradation of forage crops. *Journal of the British Grassland Society*, 28: 104-111.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74: 3583-3597.

Yang, W.Z., Beauchemin, K.A. and Rode, L.M. 1999. Effects of an enzyme feed additive on extent of degradation and milk production of lactating dairy cows. *Journal of Dairy Science*, 82: 391-403.

Animal Nutrition and Feed Technology (2013) 13: 575-582

Effect of the Addition of Enzymes on Chemical Composition and *In Vitro* Gas Production of Hybrid Maize Varieties Preserved by Silage in the Highlands[#]

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ABSTRACT

Ruiz, P.J.A., Ortiz, R.A., Peñuelas-Rivas, G., Morales, O.A., Gutierrez, M.G., Pescador P.N. and González-Ronquillo, M. 2013. Effect of the addition of enzymes on chemical composition and in vitro gas production of hybrid maize varieties preserved by silage in the highlands. Animal Nutrition and Feed Technology, 13: 575-582.

The aim of this study was to evaluate the chemical composition and *in vitro* gas production of ten varieties of corn hybrids (Owl, Copper, Chrome, H40, H47, H66, H70, HIT7, Pioneer 1832 and Victoria) cropped in the high valleys of Mexico, preserved by ensiling with three treatments, control (CTR), acetic acid 1% (AAC), or enzymes (ENZ, Sill all ® 10g/ton); samples were performed in micro-silos (n=90); After 60 days, the micro-silages were opened. The data matrix was analysed using two multivariate techniques: (i) the variables considered for the Principal Components Factor Analysis (PCFA), and (ii) Hierarchical Cluster Analysis (CA). The first multivariate technique was to reduce the information and generate major factors. Cluster analysis shows the presence of four groups with different characteristics between the groups: G1 as energy silages (H47 and Pioneer varieties), G2 protein silages (Chrome, H66, Victoria varieties), G3 easily degradable silages (Copper, HIT7 varieties), and G4 balanced silage (Owl, H40, H70 varieties). Treatments AAC and ENZ in G2, and ENZ in G3 were higher in crude protein (CP) content than the rest of the treatments. Treatments with ENZ in G1, G2, and G3 had the highest neutral detergent fiber (NDF) content ($P<0.01$). ME and NE_l were higher for G1 treated with AAC, ENZ, and CTR and G2 CTR than the rest of the treatments. The lowest pH ($P<0.01$) was for G2 and G4 treated as CTR and AAC, compared with G1 and G2 treated with AAC and ENZ. *In vitro* gas production (ml gas/g DM) was higher ($P<0.05$) for G3 and G4 treated with enzymes compared with G1 CTR and AAC. There were no differences ($P>0.05$) for *in vitro* dry matter digestibility, but NDF digestibility was higher ($P<0.01$) for G1 treated with CTR, AAC and ENZ, G2 treated with CTR, and G4 treated with ENZ than the rest of the treatments. As a conclusion,

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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the study shows depending on the focus, all the four groups of the silages i.e., energy silages (G1), protein silages (G2), easily degradable silage (G3), and balanced silage (G4) can be used in livestock feeding.

Key words: Lactic acid bacteria, Chemical composition, *In vitro* gas production.

INTRODUCTION

The corn silage is the main source of livestock feed in the center of Mexico; this has led to the implementation of programs for choosing corn varieties with higher forage production (SAGARPA, 2010). The increased demand for animal feed and the low availability of land for cultivation has necessitated the search for new varieties of hybrid maize (Johnson *et al.*, 2003; Ivan *et al.*, 2005), which implies the need for new alternatives with heterosis for increased nutritional value, both forage and grain.

The method of silage preservation is based on soluble carbohydrates converted to organic acids, mainly lactic acid, under anaerobic conditions through the lactic acid bacteria (McDonald *et al.*, 1991). To improve the silage process, chemical and biological additives have been employed (Adesogan and Salawu, 2004), which when combined with bacterial inoculants and enzymes, are one of the most commonly used for the rapid production of lactic acid, and consequently, the decrease of pH (Filya *et al.*, 2006). The technique of *in vitro* gas production (Menke and Steingass, 1988), or the modifications made by Theodorou *et al.* (1994), allow us to know the fermentation and degradation of food according to the nutritional quality and availability of nutrients for bacteria. The objectives of this study were to determine the chemical composition and *in vitro* gas production of whole plant corn silage, preserved with enzymes or chemical additives.

MATERIALS AND METHODS

Experimental site and treatments

The experiment was conducted in Toluca, State of Mexico (99°39'14" West and 19° 37'32" North). Ten corn varieties were evaluated: Owl, Copper, Chrome, H40, H47, H66, H70, HIT7, Pioneer 1832, and Victoria; which were grown in the spring-summer of 2009. Three samples were taken from each variety, grounded (General Electric mill, 390N 5KH Mod 5525; length 5 cm), and kept in micro-silos using three micro-silos per treatment: (1) control (CTR), (2) a bacterial-enzymatic compound, 10 g/ton (ENZ) (Sil All®, Alltech, *Streptococcus faecium, Lactobacillus plantarum, Pediococcus acidilactici, Lactobacillus salivarius* and enzymes cellulase, hemicellulase, pentosanase and amylase), and (3) acetic acid (AAC) 1% as a chemical preservative. The micro-silos were prepared by placing 1.5 kg of each variety of corn in a PVC tube (13 x 25 cm) and covering them with a polyethylene bag, compacting and sealing them well, and eliminating most of the oxygen present in the sample. After 60 days, the micro-silages were opened, the pHs were determined (Conductronic model pH 130);

200 g of sample were taken from each silage, dried in a forced air oven (60°C, 48 h), and ground in a Willey mill (2 mm diameter). Silage samples were analysed for dry matter (DM), ash according to AOAC (1991). Crude protein (CP), Neutral detergent fiber (NDF) acid detergent fiber (ADF), and Lignin (ADL) levels were determined by Near infrared spectroscopy (Buchi NIR FLEX N400) and NIRCal software version 4.01 (Buchi). Metabolizable energy (ME, Mj kg DM) and Net Energy Lactation (NE_L, Mj/kg DM), was determined by the equation proposed by (Menke and Steingass, 1988):

$$ME = 14.51 - (0.143 \times ADF)$$

Where, ME = (MJ/kg DM) and ADF = (g/kg DM).

In vitro gas production

In vitro gas production (GP, ml gas/g DM) and *in vitro* dry matter digestibility (DMd) were determined following the technique described by Theodorou *et al*. (1994). 800 mg of DM sample was placed in a 125 ml flask and 90 mL of incubation solution (Menke and Steingass, 1988) was added to the flask, each treatment was run in triplicate. Rumen fluid was drawn from three fistulated dairy cattle (500±20 kg LW) fed with alfalfa hay, corn stover, a concentrate (16% CP; 11.7 ME, MJ/kg DM), and a mineral supplement with *ad libitum* access to drinking water. The extracted rumen fluid was filtered thought a triple layer of gauze, then was homogenized under CO_2 for 5 min, and finally 10 ml of rumen fluid were added to each bottle and incubated (three series) in a water bath at 39°C. Gas production was recorded at 3, 6, 9, 12, 24 and 30 h using a pressure transducer (HD 8804, DELTA OMS). Additionally, three blanks per series of incubation were used (as well with three repetitions). At the end of the incubation, the residue was filtered and washed with distilled water and dried in an oven (65°C, 48 h) to determine the DMd and NDF disappearance (NDFd). Relative gas production (RGP, millilitres of gas from sample after 30 h/g DMd), was determined as in González Ronquillo *et al*. (1998). Results of gas production were fitted to the equation proposed by Krishnamoorthy *et al*. (1991):

$$GP = b\,(1 - e^{-ct})$$

Where GP = Gas production (mL gas/g DM initial); b = total gas production (ml gas/g DM); c = degradation rate with respect to the time; t = time (h).

Statistical analyses

The data of the variables obtained were initially subjected to an exploratory analysis of tests for normality and multiple correlations to determine the feasibility of the data (Hair *et al*., 1999). The variables considered for the Principal Components Factor Analysis (PCFA) are shown in Table 1.

Once the data matrix was analysed using two multivariate techniques: (i) Principal Components Factor Analysis (PCFA), and (ii) Hierarchical Cluster Analysis (CA). The first multivariate technique was to reduce the information and generate major

Table 1. Principal component analysis of 30 corn silage with 10 different corn varieties and three treatments

Rotated component matrix	Component		
	F1	F2	F3
CP	-0.23	0.87	-0.07
NDF	-0.85	-0.07	-0.07
ADF	-0.89	0.40	0.07
ADL	-0.15	0.84	0.30
ME	0.89	-0.41	-0.10
NEl	0.89	-0.41	-0.10
OM	0.26	0.26	0.84
pH	0.62	0.24	-0.59
b	-0.20	0.03	0.82
Total variance explicated			
Total	3.70	2.11	1.88
% variance	41.14	23.52	20.92
% accumulated	41.14	64.66	85.59

KMO=0.788, Extraction method, principal component analysis and rotation method: Varimax

Factor, F1 refers to the negative correlation energy content and fiber content, F2 refers to the component related to the contents of CP and ADL, F3 is related to the amount of OM and *in vitro* degradability of the corn silage.

factors. These were rotated with Varimax, which allowed to choose all data those that explanation of the variables presented (Garcia, 2008); with CA the cases were grouped according to the similarity and differences between cases, that is internally homogeneous and externally heterogeneous (Guisande *et al.*, 2006). For CA we only used the rotated factors with an eigenvalue higher than 0.5 (Hair *et al.*, 1999); the clustering method was Ward's; the measure was the Euclidean distance squared. The PCFA was performed on SPSS version 15.0; the CA was performed with the statistical software STATISTICA version 7. These new variables were used for cluster analysis to determine the presence of observations with similar characteristics. Obtained groups were analysed using SAS PROC MIXED (SAS, 1999); the model used was:

$$Y = XB + Z\gamma + \varepsilon$$

Where Y=the observed data vector; X=Fixed effect determined by the group, variety, and treatment; ß=unknown vector of fixed effects parameters analysed (group, variety, treatment) with an unknown design matrix; γ=unknown vector of random effects parameters with known design matrix (Z); ε=unknown random error vector

RESULTS AND DISCUSSION

The PCA shows three factors (F), which together account for 85.6% of the total variance as shown in Table 1; the F1 refers to the negative correlation energy content and fiber content, F2 refers to the component related to the contents of CP and ADL, F3 is related to the amount of OM and *in vitro* degradability of the corn silage.

The CA was determined with the coordinates of the rotated factors (Fig. 1), which show the presence of four groups, with different characteristics between groups. The characteristics of each group, with respect to its variables, are presented in Table 2; the groups (G) were G1 (energy silage), G2 (protein silage), G3 (Easily degradable silage), and G4 (balanced silage), in which high quality levels observed for both energy and protein degradability.

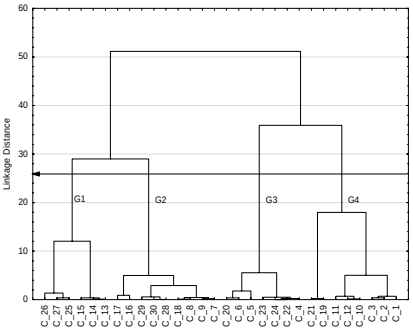

Fig. 1. Dendogram of 30 corn silage with 10 different corn varieties and three treatments, by Ward's method, square Euclidean distances.

Group 1 was characterized by higher ME and NE_l and negative CP, NDF, ADF, and ADL; this group was also the lowest number of cases presented showing the varieties H47 and Pioneer 1832 (Table 2) similar values were founded by Corral-Luna *et al*. (2011). Group 2 had the lowest b fraction and negative NDF, but in contrast, had the best performance of the CP and the highest ADL, and included the varieties Cromo, H66 and Victoria (Table 2). Kung *et al*. (1993) did not find differences in the

Table 2. Corn silages varieties derived from the cluster analyses

Energetic Silages			Protein Silages			Easily Degradable Silage			Balanced Silages		
Case	Variety	Tx	Case	Variety	Tx	Case	Variety	Tx	Case	Variety	Tx
C13,14,15	H47	CTR	C7,8,9	CHROME	CTR	C4,5,6	COPPER	CTR	C1,2,3	OWL	CTR
C25,26,27	PIONEER	CTR	C16,17,18	H66	CTR	C22,23,24	HIT7	CTR	C10,11,12	H40	CTR
C13,14,15	H47	AAC	C28,29,30	VICTORIA	CTR	C4,5,6	COPPER	AAC	C19,20,21	H70	CTR
C25,26,27	PIONEER	AAC	C7,8,9	CHROME	AAC	C22,23,24	HIT7	AAC	C1,2,3	OWL	AAC
C13,14,15	H47	ENZ	C16,17,18	H66	AAC	C4,5,6	COPPER	ENZ	C10,11,12	H40	AAC
C25,26,27	PIONEER	ENZ	C28,29,30	VICTORIA	AAC	C22,23,24	HIT7	ENZ	C19,20,21	H70	AAC
			C7,8,9	CHROME	ENZ				C1,2,3	OWL	ENZ
			C16,17,18	H66	ENZ				C10,11,12	CHROME	ENZ
			C28,29,30	VICTORIA	ENZ				C19,20,21	H70	ENZ

Values are expressed as means of different literals indicate significant difference ($P<0.05$), Tx, treatment; CTR, control group untreated; ENZ, treatment with enzymes (Sill All®); AAC, acetic acid treatment.

Table 3. Nutritional composition of four groups of silage corn under three different treatments using PROC MIXED, SAS.

N	Group	Tx	CP	NDF	ADF	ADL	ME	NE_L	OM	pH	b	c	Lag time	DMd	RGP	FNDd
6	G1	CTR	87*	519*	309*	60*	10.10*	6.05*	920*	4.64	253*	0.05	2.00	48.5	312	56.5*
6		AAC	92*	506*	312*	65*	10.04*	6.02*	936	4.78*	292*	0.04	1.41	49.6	341	57.2*
6		ENZ	89*	528*	322*	58*	9.90*	5.91*	927	4.33*	314	0.05	1.42	52.6	365	55.1*
9	G2	CTR	82	511	314	62*	10.01*	5.99*	930*	3.98*	295	0.05	1.50	56.8	378	54.3*
9		AAC	106*	540	343	64	9.60	5.71	930	4.00*	282	0.05	1.33	51.4	381	51.4*
9		ENZ	105*	555*	348*	73*	9.53	5.66	922	4.19	328	0.05	1.46	51.4	386	50.1*
6	G3	CTR	93	520	327	84*	9.82*	5.86*	936*	4.28	300	0.05	1.37	51.1	409	52.4*
6		AAC	89*	532	330	67*	9.79	5.84	934*	4.68*	338	0.04	1.45	52.6	384	50.1*
6		ENZ	109*	557*	359*	72*	9.37	5.55	928*	4.80*	310*	0.04	1.41	50.4	354	50.6*
9	G4	CTR	93*	545	341	70*	9.63*	5.73*	922*	3.88*	295	0.05	1.38	52.3	357	52.6*
9		AAC	88*	532	331	68	9.77	5.83	927	4.16*	322	0.05	1.30	53.9	398	51.4*
9		ENZ	94*	523*	332*	69*	9.75	5.81	926	4.36	321*	0.05	1.47	55.3	422	55.5*
SEM			0.27	0.86	0.61	0.55	0.01	0.01	0.05	0.02	3.42	0.00	0.05	2.07	33.7	1.20
Residual			6.65	65.34	33.35	26.51	0.01	0.001	0.26	0.05	1042	0.01	0.18	3.25	2.55	3.65
P value																
G			1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.13	0.59	0.01
Tx			0.01	0.01	0.01	0.17	0.01	0.01	0.01	0.01	0.01	0.20	0.25	0.89	0.69	0.20
G*Tx			0.05	0.01	0.01	0.21	0.01	0.01	0.02	0.02	0.05	0.67	0.65	0.13	0.59	0.01

Values are expressed as means, *, $P<0.05$;

Group: G1, Energetic silages; G2, Protein silages; G3, Easily degradable silages; G4, Balanced silages; Tx: treatments; CTR, Control group untreated; ENZ, treatment with enzymes (Sill All®); AAC, acetic acid treatment.

b, total gas production (ml gas/g DM incubated); c, range of fermentation (h); Lag time (initial fermentation time); DMd%, percentage of DM disappeared; RGP, relative gas production (mL gas/g DMd%); NDFd%, percentage of NDF disappeared.

ME, Metabolizable energy (Mj/kg DM); NE_L, Net energy lactation (Mj/kg DM).

CP content in corn silages; on the other hand, Ruiz *et al*. (2009) found differences in the CP content in different corn silages varieties and treatments. Group 3 showed the highest MO and b fraction, but had the lowest values reported for ADF and negative for CP, ADF, ME, and NE_l. This group included the varieties Cobre and Hit7; there were differences for the OM content ($P=0.01$) between treatments. Ruiz *et al*. (2009) reported differences in the OM content of different corn silage varieties, similar to the present study. Colombatto *et al*. (2003) found differences in the OM content in corn silages treated with enzymes compared with the control group, with higher values than the present study.

Table 2 Shows the four groups derived from the CA. G1 was characterized by energetic silages as H47 and Pioneer varieties; G2 present the protein silages which were Chrome, H66, and Victoria corn silages; G3 show the easy silage degradability which were Cooper and Hit7 varieties; and finally G4 show the balanced silages with the varieties Owl, H40, H70 and Chrome. There were differences between treatments ($P<0.05$) the interaction groups and treatments ($P<0.05$), Treatments AAC and ENZ in G2, and ENZ in G3 were higher in CP content than the rest of the treatments; Treatments with ENZ in G1, G2, and G3 had the highest NDF content ($P<0.01$), ME and NE_l was higher for G1 treated with AAC, ENZ and CTR and G2 CTR than the rest of the treatments, the lowest pH ($P<0.01$) was for G2 and G4 treated as CTR and AAC compared with G1 and G2 treated with AAC and ENZ; *In vitro* gas production (ml gas/g DM) was higher ($P<0.05$) for G3 and G4 treated with enzymes compared with G1 CTR and AAC. There were no differences ($P>0.05$) for *in vitro* Dry mater digestibility, but NDF digestibility was higher ($P<0.01$) for G1 treated with CTR, AAC and ENZ, G2 treated with CTR, and G4 treated with ENZ than the rest of the treatments.

CONCLUSIONS

This study shows that depending on the focus, we can use four groups of silages involving energy silages (H47 and Pioneer varieties), protein silages (Chrome, H66, Victoria varieties), easily degradable silages (Copper, HIT7 varieties) and balanced silages (Owl, H40, H70 varieties) for livestock production and feeding.

ACKNOWLEDGMENT

Mr. Ruiz Perez was granted for a CONACyT fellowship during his studies in the University Autonomous State of Mexico. We also thank Miss. Liz Hooper, LTC-University of North Texas for the critical review of the present manuscript.

REFERENCES

Adesogan, A.T. and Salawu, M.B. 2004. Effect of applying formic acid, or *Lactobacillus buchneri* inoculants with or without homofermentative lactic acid bacteria on the fermentation characteristics and aerobic stability of intercropped pea-wheat silages and whole crop wheat or pea silages. *Journal Science Food Agriculture*, 84: 983-992.

AOAC. 1991. *Official Methods of Analysis*, 15th ed.: Association of Official Analytical Chemistry INC. VA, USA.

Colombato, D.F., Mould, M.K., Bhat, R.H. and Phipps Owen, E. 2003. *In vitro* evaluation of fibrolytic enzymes as additives for maize (*Zea mays* L.) silage. II: Effects on rate of acidification, fiber degradation during ensiling and rumen fermentation. *Animal Feed Science and Technology*, 111: 129-143.

Corral-Luna, A., Dominguez-Diaz, D., Rodríguez-Almeida, F.A., Villalobos-Villalobos, G., Ortega-Gutiérrez, J.A. and Muro-Reyes, A., 2011. Composición química y cinética de degradabilidad de ensilaje de maíz convencional y sorgo de nervadura café. *Revista Brasilerira de Ciencias Aricolas*, 6: 181-187**.**

Filya, I., Sucu, E. and Karabulut, A. 2006. The effect of *Lactobacillus buchneri* on fermentation, aerobic stability and ruminal degradability of maize silage. *Journal of Applied Microbiology*, 101: 1216-1223.

Garcia, M. A. 2008. Recent dynamics of the systems of cattle in the Central Pyrenees and evaluation of their ability to adapt to the socioeconomic environment. Doctoral Thesis. University of Zaragoza. (in Spanish)

Getachew, G., Blummel, M., Makkar, H.P.S. and Becker, K. 1998. *In vitro* gas measuring techniques for assessment of nutrition quality of feeds: a review. *Animal Feed Science and Technology,* 72: 261-281.

Gonzalez Ronquillo, M., Fondevilla, M., Barrios, U.A. and Newman, Y. 1998. *In vitro* gas production from buffel grass (*Cenchrus ciliaris L*). Fermentation in relation to the cutting interval, the level of nitrogen fertilization and the season of growth. *Animal Feed Science and Technology*, 72: 19-35.

Guisande, G. C., Barreiro, F. A., Moneiro, E. I., Riveiro A. I, Vergara, C. A. R. and L. Vaamonde A. 2006. Data processing. Diaz de Santos, Spain, pp 207-273.

SAGARPA. 2010. Servicio de Información Agroalimentaria y Pesquera [en línea] http://www.siap.sagarpa.gob.mx/Consultado 20 de junio 2012.

Ivan, S.K., Grant, R.J., Weakley, D. and Beck, J. 2005. Comparison of a corn silage hybrid with high cell-wall content and digestibility with hybrid of lower cell-wall content on performance of Holstein cows. *Journal of Dairy Science,* 88: 244-254.

Johnson, L.M., Harrison, J.H., Davidson, D., Mahanna, W.C. and Shinners, K. 2003. Corn silage management: Effects of hybrid, chop length and mechanical processing on digestion and energy content. *Journal of Dairy Science*, 86: 208-231.

Krishnamoorthy, U., Soller, H. and Menke, K.H. 1991. A comparative study on rumen fermentation or energy supplements *in vitro*. *Journal of Animal Physiology Animal Nutrition*, 65: 28-35.

Kung, L. and Shaver, R. 2001. Interpretation and use of silage fermentation. Analysis reports. In: *Focus on Forage*. Wisconsin Team Forage. Vol. 3: No. 13.

McDonald, P., Henderson, A.R. and Heron, S.J.E. 1991. *The Biochemistry of Silage*. Chalcombe Publications, London, UK, 340 pp.

Menke, K.H. and Steingass, H. 1988. Stimulation of the energy fed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research Development*, 28: 7-12.

Ruiz, B.O., Castillo, Y., Anchondo, A., Rodríguez, C., Beltrán, R. and La O O y Payan, J. 2009. Efecto de las enzimas inoculantes sobre la composición del ensilaje de maíz. *Archivos de Zootecnia*. 58: 163-172.

SAS. 1999. *User's Guide: Statistics version 8*. Statistical Analysis system Institute, Cary, NC, USA.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B. and France, J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology*, 48: 185-197.

Animal Nutrition and Feed Technology (2013) 13: 583-592

Effect of a Fibrolytic Enzymatic Extract from *Cellulomonas flavigena* on *In Vitro* Degradation and *In Vivo* Digestibility and Productive Performance of Lambs[#]

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ABSTRACT

Torres, N., Mendoza, G.D., Bárcena, J.R., González, S.S., Loera, O., Salem, A.Z.M. and Lara, A. 2013. Effect of a fibrolytic enzymatic extract from Cellulomonas flavigena on in vitro degradation and in vivo digestibility and productive performance of lambs. Animal Nutrition and Feed Technology, 13: 583-592.

An enzymatic extract from *Cellulomonas flavigena* was evaluated at 0, 2.5, 7.5, 12.5 mL/kg DM of total mixed ration (TMR) on the *in vitro* degradation of DM, NDF and ADF and *in vivo* at 0, 5.0 and 7.5 mL of extract per kg DM of TMR to determine the digestibility and productive performance of lambs fed a TMR made up of 60% forage. Twenty four Pelibuey-Kathadin lambs were used in the trial. The *in vitro* degradation of ADF showed a linear ($P<0.05$) response from 6 to 72 h. There was no effect on DM intake, daily gain or feed conversion. The enzymatic dose tended to linearly decrease the apparent digestibility of DM ($P=0.06$), NDF ($P=0.10$) and ADF ($P=0.06$). The N-NH_3 concentration showed a linear decrease ($P=0.002$) and total VFA concentration was linearly ($P<0.001$) increased. The incorporation of extract of *Cellulomonas flavigena* in the diet increased *in vitro* degradation of cellulose in terms of ADF but did not increase the digestion or productive performance of lambs.

Key words: Digestibility, Feed intake, Exogenous fibrolytic enzymes, *In vitro*, Lamb.

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

Exogenous fibrolytic enzymes (EFE) are used to improve the digestibility of the cell wall in forage to increase dry matter intake and digestible energy in ruminants (Beauchemin and Holtshausen, 2010). However, the effects of commercial fibrolytic enzymes are not always consistent (McAllister *et al.*, 2001; Beauchemin *et al.*, 2003, Beauchemin and Holtshausen, 2010). Therefore, new potential alternatives derived from micro-organisms used in the production of biofuels such as *Cellulomonas flavigena* have been evaluated (Pérez-Avalos *et al.*, 2008; Rojas-Rejon *et al.*, 2011). *Cellulomonas flavigena* is a bacterium that, when cultivated in liquid fermentation, produces an enzymatic extract with xylanases and cellulases (Sánchez-Herrera *et al.*, 2007; Pérez-Avalos *et al.*, 2008; Abt *et al.*, 2010) that can hydrolyze structural carbohydrates in forage cell walls used in the feeding of ruminants (Pérez-Avalos *et al.*, 2008). According to Hernández *et al.* (2011), the enzymes of *C. flavigena* have a half-life of 23.9 h in ruminal conditions when evaluated *in vitro*, which indicates that, these enzymes are resistant to bacterial ruminal proteolysis. This has been confirmed by adding an enzyme extract of *C. flavigena* which increased the *in situ* degradability of NDF and ADF of corn stover and alfalfa hay (Hernandez, 2009). However, reports on evaluation of these extracts in animals are scarce; therefore the objective of this research was to evaluate different doses of an enzymatic extract of *C. flavigena* on *in vitro* degradation and *in vivo* digestibility and productive performance of lambs fed a total mixed ration with 60% forage (corn stover and alfalfa hay).

MATERIALS AND METHODS

The *in vitro* experiment was conducted at the Postgraduate College, Montecillo Campus, Mexico and the *in vivo* experiment at the Rancho El Trece of the Autonoma University of Chapingo in Huitzilac, Morelos, Mexico. The handling of animals was done according to the supervision of the Academic Committee of the Department of Animal Science Postgraduate College.

In vitro degradation experiment

Enzymatic extract and diet: The doses of enzymatic extract from *Cellulomonas flavigena* strain CDBB-531 tested (treatments) were 0.0, 2.5, 7.5, 12.5 mL kg per DM of TMR diluted in 240, 237.5, 232.5 and 227.5 mL of distilled water, respectively. The extract was obtained from a fermentation liquid using sugarcane bagasse as the substrate (Vega-Estrada *et al.*, 2002) and had a xylanolytic and carboxy methyl cellulolytic (CMCase) activity of 19.20 and 2.67 IU/mL, respectively (Loera and Cordova, 2003). IU was defined as the amount of enzyme that liberates 1 micromol of xylose (xylanases) or glucose (cellulases) per mL per minute. Before spraying the extract on the forage, diet ingredients were ground in a Willey mill (Arthur H. Thomas Company, Philadelphia, PA, USA) to pass through a 1 mm screen. After spraying the enzyme on the forage component of the TMR, it was mixed with the concentrate in the ratio of

60% forage and 40% concentrate. The forage component was composed of 30% corn stover and 30% alfalfa hay whereas the concentrate was made up of 15% corn, 10% sorghum, 6% soybean paste, 7% molasses, 1% urea and 1% mineral premix and was formulated according to the recommendations of the NRC (1985). The composition of the diet on a dry matter basis was (g/kg): DM 953.6, CP 156.9 (AOAC, 2005, ID954.01), NDF 429.7 and ADF 263.7 (Van Soest *et al.*, 1991).

In vitro degradation: After 16 h of application of the extract, 0.5 g of diet was weighed in ANKOM® F57 (ANKOM Technologies, Macedon, NY, USA) bags. Ruminal fluid was obtained from three Holstein bulls (450 kg BW) fitted with permanent ruminal cannula and fed 60% forage (35% oat hay and 25% corn silage) and 40% concentrate TMR with 16% crude protein and offered water *ad libitum*. The *in vitro* degradation of dry matter (IVDMD) of the diet was determined using the technique of Tilley and Terry (1963). The periods were evaluated at 6, 12, 24, 48 & 72 h in a Daisy ANKOM® model D200 (ANKOM Technologies) incubator. The *in vitro* degradation of neutral detergent fibre (IVNDFD) and acid detergent fibre (IVADFD) were determined sequentially by the analysis of residues obtained from IVDMD to determine concentrations of NDF and ADF according to the methodology of Van Soest *et al.* (1991) in a fibre analyser ANKOM® model 200 (ANKOM Technologies) using two tubes (replicates) for each incubation time; the degradation test was repeated three times.

In vivo experiment

Animals and feeding: Twentyfour Pelibuey-Kathadin lambs of 23.3±3.52 kg initial body weight (BW) were used. The lambs were randomly distributed in individual metabolic cages to evaluate increasing doses of the extract (treatments) in a completely randomized design. Before the experiment, all animals were dewormed using Ivermectin and given vitamin A, D, and E, over a 10-day for adaptation period. The TMR was fed twice daily at 08:00 h and 16:00 h and the feeding period lasted for 42 days. Feed intake (kg/d DM) was recorded daily.

Treatments: The treatments were equivalent doses of 95.0 and 142.5 IU/mL of xylanolytic activity extract per kg DM of TMR i.e. 0 mL, 5.0 and 7.5 mL of extract. The extract was diluted in 240 mL of distilled water and the solution was sprayed on the forage component of the TMR before mixing it with concentrate component of the diet prior to feeding the lambs.

Ruminal fermentation, digestibility and productive performance: A sample of 50 mL of ruminal fluid was collected from each lamb using an esophageal probe on the last day of the experiment. The pH was measured immediately and the rest of the ruminal fluid was preserved after acidification with 2 mL of 25% metaphosphoric acid. The samples were kept frozen (-20°C) until the analysis of VFA by gas chromatography (Erwin *et al.*, 1961) and N-NH_3 by spectrophotometry (McCullough, 1967).

Each lamb was weighed every 14 days after 12 h of fasting to estimate the average daily gain (ADG, g/d) and feed conversion. On day 20 to 24, faeces were collected from each animal for five consecutive days to determine the DM digestibility using acid insoluble ash as an internal marker (Keulen and Young, 1977). The digestibility of NDF and ADF (Van Soest *et al.*, 1991) were also determined.

Statistical analysis

The results of IVDMD, IVNDFD and IVADFD were analysed according to a completely randomised design using the GLM program of SAS 9.0 (2002). Polynomial non-orthogonal contrasts were used to test linear and quadratic effects of the enzymes. The coefficients of the non-orthogonal contrasts were estimated with the IML program (SAS 9.0, 2002). The means were compared with the Tukey test (Steel and Torrie, 1986).

The results of the ruminal fermentation, digestibility and productive performance were analysed as a completely randomized design using the GLM program in SAS 9.0 (2002). Polynomial non-orthogonal contrasts were used to test the linear and quadratic effects of the enzymes. The non-orthogonal coefficients were estimated with the IML program in SAS 9.0 (2002).Means were also compared with Tukey test (Steel and Torrie, 1986).

RESULTS AND DISCUSSION

In vitro degradation

The IVDMD showed a linear response ($P<0.05$) with an increase of the enzymatic extract dose (Table 1), presenting a greater degradation at 6 h of incubation. The IVNDFD did not change as a result of the extract from 6 to 24 h of incubation (Table 1). At 48 h, the increase in enzymatic dose tended to cause a quadratic decrease ($P=0.06$) in the IVNDFD. The increase in the dose of the extract linearly affected ($P=0.01$) the IVADFD (Table 1) from 6 to 72 h of incubation of the diet.

Although some exogenous enzymes did not improve the IVDMD (Avellaneda-Cevallos *et al.*, 2009; González-García *et al.*, 2010), the linear response in the IVDMD at 6 h of incubation as a result of the enzymatic extract from *Cellulomonas flavigena* determined in this study agree with that reported by Pinos *et al.* (2001) and Moreno *et al.* (2007); these groups incubated alfalfa hay and a diet with 40% of the same forage added with 2 g/kg DM of xylanases from *Aspergillus niger* and *Trichoderma viride*. The results of this experiment confirm that the exogenous enzymes stimulate the initial phase of degradation of the substrate (Moreno *et al.*, 2007; Giraldo *et al.*, 2008a).

However, the IVNDFD from 6 to 24 h of incubation of the diet contrasts with that reported by Eun *et al.* (2007) and Moreno *et al.* (2007), when they added endoglucanases and commercial xylanases to alfalfa hay and to a diet with 50% of the

same forage during the first 24 h of incubation. The tended quadratic decrease (P=0.06) in the IVNDFD observed at 48 of incubation could be due to a decrease in ruminal pH, which was generated by the greater availability of non-structural carbohydrates (Grant, 1994; González-García *et al.*, 2010). The effect observed in IVDMD and IVNDFD confirm that the response in ruminal digestibility to exogenous fibrolytic enzymes can be variable depending on the type and quantity of enzymes, as well as the enzyme-substrate interaction and the forage: concentrate proportion (McAllister *et al.*, 2001; Beauchemin *et al.*, 2003; Giraldo *et al.*, 2008a).

Table 1. Degradation coefficient (g digested/g incubated) *in vitro* of DM, NDF and ADF of the total mixed ration (TMR) containing 60% of forage added with extract of *Cellulomonas flavigena*

Incubation	Dose, mL/kg DM of TMR				SEM†	P‡	
time, h	0	2.5	7.5	12.5		Linear	Quadratic
DM							
6	0.289^{d}	0.297^{cd}	0.287^{d}	0.302^{c}	0.002	0.03	0.08
12	0.449	0.448	0.450	0.450	0.011	0.91	0.99
24	0.565	0.552	0.561	0.555	0.009	0.67	0.88
48	0.711	0.683	0.683	0.685	0.013	0.28	0.29
72	0.746	0.736	0.742	0.745	0.008	0.88	0.58
NDF							
6	0.272	0.283	0.263	0.258	0.016	0.43	0.84
12	0.494	0.480	0.483	0.468	0.019	0.46	0.97
24	0.519	0.515	0.538	0.503	0.010	0.58	0.13
48	0.807	0.787	0.778	0.801	0.009	0.72	0.06
72	0.762	0.717	0.766	0.773	0.013	0.12	0.30
ADF							
6	0.293	0.321	0.370	0.403	0.022	0.009	0.69
12	0.347^{e}	0.374^{de}	0.420^{cd}	0.473^{c}	0.013	0.0004	0.89
24	0.490^{d}	0.506^{d}	0.566^{c}	0.592^{c}	0.009	0.0001	0.37
48	0.640	0.627	0.673	0.686	0.017	0.03	0.91
72	0.689^{d}	0.739^{c}	0.730^{c}	0.732^{c}	0.007	0.02	0.03

†Standard error of the mean

‡Probability of a significant effect of enzyme dose (linear or quadratic effect)

^{cde}Means with different superscript letters within rows are different (P<0.05)

The linear effect in the IVADFD due to the enzymatic extract doses observed from 6 to 72 h of incubation of the diet can be explained by the cellulolytic activity of the enzymatic extract (Sánchez-Herrera *et al.*, 2007; Abt *et al.*, 2010) in the hydrolysis of the cell wall; this could have released soluble carbohydrates (Perez-Avalos *et al.*, 2008) from the forage of the diet that could adversely affect the IVNDFD at 48 h of incubation.

Productive performance, digestibility and ruminal variables

There was no effect of different doses of enzyme extracts (P>0.05) on the final BW, DM intake, ADG and feed conversion (Table 2). An increased dose of the extract tended to linearly decrease the apparent digestibility of DM (P=0.06), NDF (P=0.10)

and ADF (P=0.06) (Table 2). An increase in the dose of the extract linearly decreased (P=0.002) the N-NH_3 level, caused a quadratic response (P<0.05) in the proportion of butyrate and linearly increased (P<0.05) the total VFA concentration (Table 3) in ruminal fluid of lambs.

Table 2. Effect of an enzymatic extract of *Cellulomonas flavigena* on productive performance and the digestibility of the total mixed ration (TMR) consumed by lambs

Items	Dose, mL/kg DM of TMR			SEM†	P‡	
	0	5.0	7.5		Linear	Quadratic
Productive performance						
Initial BW (kg)	23.1	23.6	23.2	1.30	0.92	0.77
Final BW (kg)	32.4	32.6	32.0	1.43	0.89	0.78
Intake (g DM/d)	1134	1169	1143	53.56	0.84	0.67
ADG (g/d)	220	216	209	10.60	0.49	0.78
Feed conversion	5.22	5.61	5.67	0.32	0.30	0.82
Apparent digestibility, (g/kg)						
DM	696.9^{c}	644.9^{d}	672.4^{cd}	11.8	0.06	0.03
NDF	610.0	550.9	571.7	19.4	0.10	0.18
ADF	585.2	533.1	526.7	23.1	0.06	0.65

†Standard error of the mean

‡Probability of a significant effect of enzyme dose (linear or quadratic effect)

^{cd}Means with different superscript letters within rows are different (P<0.05)

Table 3. Effect of an enzymatic extract of *Cellulomonas flavigena* on ruminal variables in lambs

Items	Dose, mL/kg DM of TMR			SEM†	P‡	
	0	5.0	7.5		Linear	Quadratic
pH	7.0	6.8	6.9	0.09	0.33	0.31
N-NH_3 (mg/dL)	12.7^{c}	10.1^{cd}	7.8^{d}	1.00	0.002	0.59
Volatile fatty acids (mol/100 mol)						
Acetic	73.5	74.3	75.6	0.81	0.58	0.19
Propionic	15.2	16.7	16.4	0.89	0.28	0.53
Butyric	11.3^{c}	9.1^{d}	11.0^{cd}	0.60	0.38	0.01
Total VFA (mM/L)	37.4^{d}	46.0^{cd}	56.7^{c}	3.17	0.0004	0.30

†Standard error of the mean

‡Probability of a significant effect of enzyme dose (linear or quadratic effect)

^{cd}Means within rows different superscript letters are different (P<0.05)

The productive performance of lambs was similar to what has been observed in other experiments (Giraldo *et al.*, 2008b; Pinos-Rodríguez *et al.*, 2008; Almaraz *et al.*, 2010) that used exogenous enzymes without observing changes in intake, ADG or feed conversion. In contrast, the results of this experiment are different as 24% and 19% average increases in ADG and feed conversion, respectively, were reported by Cruywagen and Goosen (2004) and Cruywagen and van Zyl (2008) in lambs fed diets

with 60% forage supplemented with enzymes from *Aspergillus terreus* var. Carneus with doses 3.4 times higher than the xylanolytic activity used in this experiment. Similarly, Gado *et al.* (2011) and Salem *et al.* (2011, 2012) also reported a higher ADG and digestibility with improved conversion in lambs receiving a commercial enzymatic product from rumen anaerobic bacteria and with a dose 5 times lower in xylanolytic activity than that used in this experiment, but also including cellulases, amylases and proteases; this suggests that the variability in productive performance in ruminants not only depends on the type and enzyme activity (Beauchemin *et al.*, 2003), but also the stability of the enzymes in the rumen (Hristov *et al.*, 1998) and the physiochemical characteristics of cell wall of forage of the diet (Jalilvand *et al.*, 2008).

Even though a fibrolytic enzymatic extract from *Cellulomonas flavigena* has shown increased *in situ* digestibility of NDF and ADF (Hernandez, 2009), in the current experiment, there was a tended linear decrease in the *in vivo* digestibility of these fractions, indicating that there may have been a negative effect on the ruminal conditions or the fibrolytic microbial populations in the rumen (Wang *et al.*, 2001; Nsereko *et al.*, 2002), probably due to the release of soluble carbohydrates (Krausse *et al.*, 2003; Wang *et al.*, 2004) from the forage (Berthiaume *et al.*, 2010) and other components of the diet. The mechanism by which the enzymatic extract of *C. flavigena* decreased the degradation of feed in lambs is unknown. However, the CMCases and xylanases present in the enzymatic extract (Sánchez-Herrera *et al.,* 2007; Pérez-Avalos *et al.,* 2008; Abt *et al.,* 2010) could have contributed to an increase in carbohydrates that are easily degraded by rumen micro-organisms. This could generate carbon catabolite repression (Forero and Sanchez, 2008), both in rumen bacteria (Moat *et al.*, 2003) and fungi (Suto and Tomita, 2001), which could inhibit structural gene transcription associated with the use of secondary carbon sources (Moat *et al.*, 2003; Forero and Sanchez, 2008). Additionally, the existence of chitinases (Reguera and Leschine, 2001; Fleuri and Sato, 2005; Abt *et al.*, 2010) and endo-1,3-β-D-glucosidases (Tang-Yao *et al.*, 2002; Fleuri and Sato, 2005) has been reported in an enzymatic extract produced by species of the genus *Cellulomonas*, which could also affect chitin and 1,3-β-D-glucans in the cell walls of ruminal fungi, thus contributing to the lower digestibility of nutrients.

The lack of response in terms of pH and the proportions of acetic and propionic acid with an increase in the dose of enzymatic extract of *C. flavigena* contrasts with results reported by Pinos-Rodríguez *et al.* (2002) and Gado *et al.* (2011), who observed higher VFA production in lambs due to enhanced fibre digestibility of the diet. Leng (1993) reported that, during ruminal fermentation, when there is no deficit of NH_3, the carbon flux is oriented towards greater capture of available carbon for microbial protein synthesis. However, the low concentration of NH_3 and the greater amount total VFA obtained with an increase in enzymes in this experiment suggest that supplementation with this extract could favour the carbon flow and VFA production and reduce for microbial protein synthesis.

CONCLUSION

The extract from *Cellulomonas flavigena* increases *in vitro* degradation, principally of the cellulose from forages such as corn stover and alfalfa hay that are used in ruminants feeding. The addition of an enzymatic extract of *Cellulomonas flavigena* to forage in the diet does not improve digestion or productive performance in lambs at the doses evaluated. Future research is necessary to determine the possible activity of chitinases and proteases in the enzymatic extract of *Cellulomonas flavigena* strain CDBB-531 and the effect of these enzymes along with xylanases and cellulases in ruminal micro-organism populations.

REFERENCES

Abt, B., Foster, B., Lapidus, A., Clum, A., Sun, H., Pukall, R., Lucas, S., Glavina, T., Rio, D., Nolan, M., Tice, H., Cheng, J.F., Pitluck, S., Liolios, K., Ivanova, N., Mavromatis, K., Ovchinnikova, G., Pati, A., Goodwin, L., Chen, A., Palaniappan, K., Land, M., Hauser, L., Chang, Y.J., Jeffries, C.D., Rohde, M., Göker, M., Woyke, T., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P., Kyrpides, N.C. and Klenk, H.P. 2010. Complete genome sequence of *Cellulomonas flavigena* type strain (134T). *Standards in Genomic Sciences*, 3:15-25.

Almaraz, I., González, S.S., Pinos-Rodríguez, J.M. and Miranda, L.A. 2010. Effects of exogenous fibrolytic enzymes on *in sacco* and *in vitro* degradation of diets and on growth performance of lambs. *Italian Journal of Animal Science,* 9: 6-9.

AOAC. 2005. *Official Methods of Analysis*, 18th ed. Association of Official Analytical Chemists, Washington, DC.

Avellaneda-Cevallos, J.H., Montañez-Valdez, O.D., González-Muñoz, S., Pinos-Rodríguez J., Bárcena-Gama, R. and Hernández-Garay, A. 2009. Effect of exogenous fibrolytic enzymes on dry matter and cell wall *in vitro* digestibility of Guinea grass hay. *Journal of Applied Animal Research,* 36: 199-202.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P. and Yang, W.Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science,* 81: E37-47.

Beauchemin, K.A. and Holstshausen, L. 2010. Developments in enzyme usage in ruminants. In: *Enzymes in Farm Animal Nutrition,* 2nd ed. (Ed. M.R. Bedford and G.G. Partridge), CABI Publishing, UK, pp. 206-230.

Berthiaume, R., Benchaar, C., Chaves, A.V., Tremblay, G.F., Castonguay, Y., Bertrand, A., Belanger, G., Michaud, R., Lafreniere, C., McAllister, T.A. and Brito, A.F. 2010. Effects of nonstructural carbohydrate concentration in alfalfa on fermentation and microbial protein synthesis in continuous culture. *Journal of Dairy Science,* 93: 693-700.

Cruywagen, C.W. and Goosen, L. 2004. Effect of an exogenous fibrolytic enzyme on growth rate, feed intake and feed conversion ratio in growing lambs. *South African Journal of Animal Science,* 34 (Suppl. 2): 71-73.

Cruywagen, C.W. and van Zyl, W.H. 2008. Effects of a fungal enzyme cocktail treatment of high and low forage diets on lamb growth. *Animal Feed Science and Technology,* 145: 151-158.

Erwin, E.S., Marco, G.T. and Emery, E.M. 1961. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. *Journal of Dairy Science*, 44: 1768-1771.

Eun, J.S., Beauchemin, K.A. and Schulze, H. 2007. Use of Exogenous fibrolytic enzymes to enhance *in vitro* fermentation of alfalfa hay and corn silage. *Journal of Dairy Science*, 90: 1440-1451.

Forero, A. and Sánchez, S. 2008. Represión catabólica por carbono de bacterias gram-positivas: inteligencia alimenticia. *Bio Tecnología*, 12: 24-48.

Fleuri, L.F. and Sato, H.H. 2005. Producción, purificación, clonación y la aplicación de las enzimas líticas. *Química Nova*, 28: 871-879.

Gado, M.H., Salem, A.Z.M., Odongo, N.E. and Borhami, B.E. 2011. Influence of exogenous enzymes ensiled with orange pulp on digestion and growth performance in lambs. *Animal Feed Science and Technology,* 165: 131-136.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. and Carro, M.D. 2008a.Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage: concentrate ratios. *Animal Feed Scienceand Technology*, 141: 306-325.

Giraldo, L.A., Tejido, M.L., Ranilla, M.J., Ramos, S. and Carro, M.D. 2008b. Influence of direct-fed fibrolytic enzymes on diet digestibility and ruminal activity in sheep fed a grass hay-based diet. *Journal of Animal Science,* 86: 1617-1623.

González-García, E., Albanell, E., Caja, G. and Casals, R. 2010. *In vitro* fermentative characteristics of ruminant diets supplemented with fibrolytic enzymes and ranges of optimal endo-β-1,4-glucanase activity. *Journal of Animal Physiology and Animal Nutrition*, 94: 250-263.

Grant, R.J. 1994. Influence of corn and sorghum starch on the *in vitro* kinetics of forage fibre digestion. *Journal of Dairy Science,* 77: 1563-1569.

Hernández, G.P.A. 2009. *Caracterización del extracto enzimático xilanolítico exógeno de Cellulomonas flavigena en condiciones ruminales*. Doctor en Ciencias, disertación. Área de Nutrición de Rumiantes, Colegio de Postgraduados, México.

Hernández, P.A., Bárcena, J.R., Mendoza, G.D., Montes, M.C., González, S.S. and Rojo, R. 2011. Xylanase activity from *Cellulomonas flavigena* extracts as affected by temperature and its degradation under *in vitro* ruminal conditions. *African Journal of Microbiology Research*, 5: 961-964.

Hristov, N.A., McAllister, T.A. and Cheng, K.J. 1998. Stability of exogenous polysaccharide-degrading enzymes in the rumen. *Animal Feed Science and Technology,* 76: 161-168.

Jalilvand, G., Odongo, N.E., López, S., Naserian, A., Valizadeh, R., Eftekhar-Shahrodi F., Kebreab, E. and France, J. 2008. Effects of different levels of an enzyme mixture on *in vitro* gas production parameters of contrasting forages. *Animal Feed Science and Technology,* 146: 289-301.

Keulen, J.V. and Young, B.A. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *Journal of Animal Science,* 44: 282-287.

Krause, D.O., Denman, S.E., Mackie, R.I., Morrison, M., Rae, A.L., Attwood, G.T. and McSweeney, C.S. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiology Reviews*, 27: 663-693.

Leng, R.A. 1993. Quantitative ruminant nutrition- A green science. *Australian Journal of Agricultural Research*, 44: 363-80.

Loera, O. and Córdova, J. 2003. Improvement of xylanase production by a parasexual Cross between *Aspergillusniger* strains. *Brazilian Archives of Biological Technology,* 46: 177-181.

McAllister, T.A., Hristov, A.N., Beauchemin, K.A., Rode, L.M. and Cheng, K. Jr. 2001. Enzymes in ruminants diets. In: *Enzyme in Farm Animal Nutrition* (Eds. M.R. Bedford and G.G. Partridge), CABI Publishing, UK, pp. 273-298.

McCullough, H. 1967. The determination of ammonia in whole blood by direct colourimetricmethod. *Clinical Chemistry*, 17: 297-304.

Moat, A.G., Foster, J.W. and Spector, M.P. 2003. Regulation of prokaryotic gene expression. In: *Microbial Physiology*, 4th ed. Wiley-Liss, Inc., New York, USA, pp. 194-238.

Moreno, R., Pinos-Rodríguez, J.M., González, S., Álvarez, G., García, J.C. Mendoza, G. and Bárcena, R. 2007. Effect of exogenous fibrolytic enzymes on *in vitro* ruminal degradation of diets for dairy cows. *Interciencia*, 32: 850-853.

NRC. 1985. *Nutriment Requirements of Sheep*, 6th ed. National Academy Press, Washington, DC, USA.

Nsereko, V.L., Beauchemin, K.A., Morgavi, D.P., Rode, L.M., Furtado, A.F., McAllister, T.A., Iwaasa, A.D., Yang, W.Z. and Wang Y. 2002. Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. *Canadian Journal of Microbiology*, 48: 14-20.

Pérez-Avalos, O., Sánchez-Herrera, L.M., Salgado, L.M. and Ponce-Noyola, T. 2008. A bifunctionalendoglucanase/endoxylanase from *Cellulomonas flavigena* with potential use in industrial processes at different pH. *Current Microbiology*, 57: 39-44.

Pinos, J.M., González, S.S., Mendoza, G., Bárcena, R. and Cobos, M. 2001. Effect of fibrolytic enzymes glycosylated *in vitro* digestibility of DM and OM from alfalfa (*Medicagosativa*) and ryegrass (*Loliumperenne*). *Revista de la Facultad de Agronomía (LUZ)*, 18: 505-509.

Pinos-Rodríguez, J.M., González, S.S., Mendoza, G.D., Bárcena, R., Cobos, M.A., Hernández, A. and Ortega, M.E. 2002. Effects of exogenous fibrolytic enzyme on ruminal fermentation and digestibility of alfalfa and rye-grass hay fed to lambs. *Journal of Animal Science,* 80: 3016-3020.

Pinos-Rodríguez, J.M., Moreno, R., González, S.S., Robinson, P.H., Mendoza, G. And Álvarez, G., 2008. Effects of exogenous fibrolytic enzymes on ruminal fermentation and digestibility of total mixed rations fed to lambs. *Animal Feed Science and Technology,* 142: 210-219.

Reguera, G. and Leschine, S.B. 2001. Chitin degradation by cellulolytic anaerobes and facultative aerobes from soils and sediments. *FEMS Microbiology Letters*, 204: 367-374.

Rojas-Rejón, O.A., Poggi-Varaldo, H.M., Ramos-Valdivia, A.C., Martínez-Jiménez, A. Cristiani-Urbina, E., de la Torre, M. and Ponce-Noyola, T. 2011. Production of cellulases and xylanases under catabolic repression conditions from mutant PR-22 of *Cellulomonas flavigena*. *Journal of Industrial Microbiology and Biotechnology*, 38: 257-264.

Salem, A.Z.M., El-Adawy, M.M., Gado, H., Camacho, L.M., González-Ronquillo, M., Alsersy, H. and Borhami, B. 2011. Effects of exogenous enzymes on nutrients digestibility and growth performance in sheep and goats. *Tropical and Subtropical Agroecosystems*, 14: 867-874.

Salem, A.Z.M., Hassan, A.A., Khalil, M.S., Gado, H.M., Alsersy, H. And Simbaya, J. 2012. Effects of sun-drying and exogenous enzymes on nutrients intake, digestibility and nitrogen utilization in sheep fed *Atriplexhalimus* foliage. *Animal Feed Science and Technology,* 171: 128-139.

Sánchez-Herrera, L.M., Ramos-Valdivia, A.C., de la Torre, M., Salgado, L.M. and Ponce-Noyola, T. 2007. Differential expression of cellulases and xylanases by *Cellulomonas flavigena* grown on different carbon sources. *Applied Microbiology and Biotechnology*, 77: 589-595.

SAS, 2002.*SAS User's Guide: Statistics* (Release 8.02). SAS Inst., Inc., Cary, NC, USA.

Steel, G.R. and Torrie J.H. 1986. *Bioestadística: Principios y Procedimientos*, 2nd ed. McGraw Hill, México.

Suto, M. and Tomita, F. 2001. Induction and catabolite repression mechanisms of cellulase in fungi. *Journal of Bioscience and Bioengineering*, 92: 305-311.

Tang-Yao, H., Chun-Wei, Ch. And Jenn-Wen, H. 2002. Isolation and biochemical characterization of an endo-1,3-*b*-glucanase from *Streptomyces sioyaensis* containing a C-terminal family 6 carbohydrate-binding module that binds to 1,3-*b*-glucan. *Microbiology*, 148: 1151-1159.

Tilley, M.A. and Terry, R.A. 1963. A two-stage technique for the *in vitro* digestion of forage crops. *Journal of the British Grassland Society*, 18: 104-109.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fibre, neutral detergent fibre, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74: 3583-3592.

Vega-Estrada, J., Flores-Contreras, L.B., Santiago, A., Magaña-Plaza, I. and Montes-Horcasitas, C. 2002. Draw-fill batch culture mode for production of xylanases by *Cellulomonas flavigena* on sugar cane bagasse. *Applied Microbiology and Biotechnology*, 58: 435-438.

Wang, Y., McAllister, T.A., Rode, L.M., Beauchemin, K.A., Morgavi, D.P., Nsereko, V.L., Iwaasa, A.D. and Yang, W. 2001. Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the rumen simulation technique (Rusitec). *British Journal of Nutrition*, 85: 325-332.

Wang, Y., Spratling, B.M., ZoBell, D.R., Wiedmeier, R.D. and McAllister, T.A. 2004. Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. *Journal of Animal Science,* 82: 198-208.

Animal Nutrition and Feed Technology (2013) 13: 593-599

Chemical Composition and *In Vitro* Gas Production from Different Varieties of Native and Hybrid Maize Silage with the Addition of Acetic Acid or Enzymes[#]

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ABSTRACT

Ruiz, P.J.A., Moreno, A.J., Salem, A.Z.M., Castelan Ortega, O. and Gonzalez-Ronquillo, M. 2013. Chemical composition and in vitro gas production from different varieties of native and hybrid maize silage with the addition of acetic acid or enzymes. Animal Nutrition and Feed Technology, 13: 593-599.

The aim of this study was to evaluate and compare the chemical composition and *in vitro* gas production of corn white local native (WLN) corn yellow local native (YLN) and the hybrids H-51EA and CL080001 as silage, preserved by three treatments, control (CTR), the addition of acetic acid (AAC) or enzymes (ENZ). Samples were prepared in microsilos and analysed in 4x3 factorial design with three replicates of each one. The dry matter content (g/kg) was higher ($P<0.01$) for CLO80001 and lower for YLN (222 vs 176); organic matter (OM) content was higher ($P<0.01$) CLO80001 compared with the natives. Regarding treatments, OM in ENZ were higher ($P<0.01$) with respect to AAC and CTR; crude protein (CP) content differs by variety and treatments, WLN variety was higher ($P<0.01$) and the lowest CP was for CLO80001. CTR and AAC were higher in CP ($P<0.01$) than ENZ treatment. Neutral detergent fiber and acid detergent fiber content was higher ($P<0.01$) for WLN than YLN and the hybridsThe highest gas production (ml gas/g DM) ($P<0.01$) was for hybrids compared with local corn natives. There were no differences ($P>0.05$) for rate c and lag time between varieties. Dry matter disappeared (DMd) was higher ($P<0.01$) for CLO8001 and WLN than H51EA. ME (MJ/kg DM) was higher ($P<0.01$) for CLO80001 < H51EA < YLN < WLN. ME were higher ($P<0.01$) in ENZ and AAC than CTR. The WLN variety proves to be the best option for feeding cattle, as it turned out better than the rest of the varieties tested. Addition of corn silage with acetic acid or enzymes increased NDF digestibility and ME availability.

Key words: Enzymes, Acetic acid, Chemical composition, *In vitro* gas production, Silage.

[#]This paper is part of the special issue entitled: *Exogenous Enzymes in Animal Nutrition - Benefits and Limitations*, Guest Edited by A.Z.M. Salem and N. Odongo, and Editor for Animal Nutrition and Feed Technology, A.K. Pattanaik.

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INTRODUCTION

Corn silage is the main source of livestock feed in the center of Mexico; this has led to implement programs for choosing corn varieties with higher forage production (SIAP-SAGARPA, 2010). The increased demand for animal feed and the low availability of land for cultivation has necessitated the search for new varieties of hybrid maize (Johnson *et al.*, 2003; Ivan *et al.*, 2005), which implies the need for new alternatives with heterosis for increased nutritional value in both forage and grain. The method of silage preservation is based on converting the soluble carbohydrates in organic acids, mainly lactic acid, under anaerobic conditions by lactic acid bacteria (McDonald *et al.*, 1991). The technique of *in vitro* gas production (Menke and Steingass, 1988), or the modifications by Theodorou *et al.* (1994) in simulating the digestive processes generated from microbial production (Getachew, 1998), allows us to know the fermentation and degradation of food according to the nutritional quality and availability of nutrients for ruminal bacterias. The objectives of this study were to determine the chemical composition and *in vitro* gas production of corn silage, preserved without additive or the addition of enzymes or chemical acids.

MATERIALS AND METHODS

Experimental site

The study was conducted in Toluca, State of Mexico (99° 39'14" West and 19° 37'32" North). Four varieties of corn were evaluated, White local native (WLN), Yellow local native (YLN), and the hybrids H-51 AE and CL080001, which were grown in the spring-summer 2009.

Three samples (10 kg Fresh matter) were taken from each variety, grounded (General Electric mill, 390N 5KH Mod 5525; length 5 cm) and kept in microsilos by triplicate, as untreated control (CTR), with a bacterial-enzymatic compound, 10 g/ton (ENZ) (Sil All$^{4x4®}$, Alltech, which contains: *Streptococcus faecium, Lactobacillus plantarum, Pediococcus acidilactici* and *Lactobacillus salivarius* and enzymes cellulase, hemicellulase, pentosanase and amylase, and to another microsilo (n=3) was added acetic acid (AAC) 1%. The microsilos were prepared by placing 1.5 kg of corn in a PVC tube (13x25 cm) and covering them with a polyethylene bag, compacting and sealing them well and eliminating most of oxygen present in the sample. After two months samples were opened and the pHs were determined (Conductronic model pH130). A part of the samples were dried (60°C, 48 h) and ground (1 mm diameter) to determine the dry matter (DM) and organic matter content (OM) (AOAC, 1991). The concentration of crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin was determined by infrared spectrophotometry using a spectrophotometer (Buchi NIR FLEX N400) NIRCal software version 4.01 (Buchi); metabolizable energy (ME, MJ/kg DM) was determined by the equation proposed by Menke and Steingass (1988):

ME (MJ/kg DM)= 14.51- (0.143xADF); *where* ME= (MJ/kg DM) and ADF= (g/kg DM).

For *in vitro* gas production technique, we used three rumen fistulated non lactating dairy cattle (LW 450±20 kg) as donors of rumen fluid. The animals received a diet of oat hay and alfalfa hay (ratio of 50:50), formulated to meet all of their nutrient requirements (NRC, 2001), and were supplied twice a day (8:00 and 16:00 hours). Fresh water was available to cows at all times.

In vitro gas production

Gas production was determined in 125 ml amber flask per triplicate and three series of incubation for each sample of forage conservation method, using the technique proposed by Theodorou *et al.* (1994). In each flask we introduced 0.8 g DM of each of the samples, then we added 90 ml of buffer solution (Menke and Steingass, 1998) gassed with CO_2 and stored (4°C for 12 h) until the next day. Then from the flasks we took 700 ml of ruminal fluid and 300 g of solid rumen contents of each donor cattle; subsequently, the homogenized mixture was filtered through four layers of gauze and glass wool; we maintained the rumen fluid at 39°C; it was gassed with CO_2, and subsequently, we added to each flask 10 ml of ruminal fluid. Finally, the flasks were introduced into a water bath at 39°C and we initiated the gas production record using a pressure transducer (DELTA OHM, Manometer, 8804). The volume of gas produced was recorded at 3, 6, 9, 12, 24 and 30 h incubation. Additionally, three blanks per series were used (as well with three repetitions).

After the incubation period (30 h), the accumulated gas was released and the fermentation residues of each flask were dried (60°C, 48 h) to calculate the proportion of dry mater disappeared (DMd, %) and relative gas production (RGP, ml gas g DMd) according to Gonzalez Ronquillo *et al.* (1998).The kinetics of gas production were determined by adjusting the model: $GP=b\ (1-e^{-ct})$ proposed by Krishnamoorthy *et al.* (1991); according to the model, *b* represents the total production of gas (ml gas/g initial DM); *c* the rate of degradation in relation to time (h); t represents the lag time (h), which is the time when the food begins to be degraded by micro-organisms in the rumen.

Statistical analysis

Samples were analysed in a completely randomized design with a 4 x 3 factorial arrangement, with varieties (4) and treatments (3), with three replicates each one (n=36), using an analysis of variance (SAS, 1999). The averages of each variable were compared with the Tukey test at $P<0.05$.

RESULTS AND DISCUSSION

There were no significant differences ($P>0.05$) for pH between varieties and treatments (Table 1); these results are different from those presented by Ruiz *et al.* (2009) and Filya *et al.* (2006), which evaluated corn silage inoculants, showing

differences in pH between treatments, with pH lower than the present study. Similarly Kristensen *et al.* (2010) found differences in pH by treatment, with similar values than those of the present study. Kung and Shaver (2001) found that corn silage rarely has a pH above 4.2, which can be associated with very dry silage (over 42% dry), and pH 3.7 to 4.2 propose a normal range of 30 to 40% dry matter. Colombatto *et al.* (2003) found pH values below 4.0 in corn silage treated with enzymes in relation to other untreated silages, due to increased substrate available for fermentation. DM content was higher ($P<0.01$) for CLO80001, and lowest for YLN; Ruiz *et al.* (2009) found no differences in DM content in different corn hybrids, with similar contents to those of the present study. Colombatto *et al.* (2003) found no differences in DM content of corn silage treated with enzymes with higher values than the present study (324 to 347 vs 176 to 222 g/kg DM). Ranjit *et al.* (2002) reported differences in DM content of corn silage with four doses of Lactobacillus buchneri 40788 compared with a control group. OM content was higher ($P=0.01$) for CLO80001 compared to corn natives; Ruiz *et al.* (2009) reported differences in OM content per treatment, with similar results to those obtained in the present study; Colombatto *et al.* (2003) found differences in OM content of corn silage treated with enzymes in relation to other corn untreated, but with higher values than the present study (943 to 952 vs 913 to 933, g/kg DM respectively). OM content was higher ($P<0.01$) for ENZ compared to AAC and CTR. CP content differs ($P<0.01$) by variety and treatments, with a higher WLN ($P<0.01$) and lower for CLO80001; CTR and AAC treatments were higher ($P<0.01$) than ENZ. Ruiz *et al.* (2009) reported differences in the CP content in treatments and varieties, with similar results to those obtained in the present study. The content of NDF and ADF was higher ($P<0.01$) for WLN compared with YLN, and higher in hybrids (CLO80001 and H51EA). Kung *et al.* (1993) show that CP, NDF and ADF were similar for all corn silage treated with two inoculants; on the contrary Ruiz *et al.* (2009) found differences in CP, NDF and ADF per variety and treatment, while Ranjit *et al.* (2002) founded differences in NDF and ADF content in corn silage with four doses of *Lactobacillus buchneri* 40788 compared with a control group. This is due to increased use of the more soluble fraction of the corn plant in relation to fibrous fractions by the ENZ.

Table 1. Chemical composition (g/kg DM) of corn silage (native and hybrid), preserved without additive (CTR) or with the addition of enzymes (ENZ) or acetic acid (ACC).

Item	VARIETY (*V*)				TREATMENT (*T*)			SEM	P value		
	YLN	CLO80001	H51EA	WLN	CTR	ENZ	ACC		*V*	*T*	*VxT*
pH	3.96	3.97	4.18	3.96	4.04	4.03	4.00	0.12	0.58	0.97	0.06
DM	176^{f}	222^{d}	185^{e}	185^{e}	171^{e}	203^{d}	202^{d}	0.52	0.01	0.01	0.01
OM	913^{e}	933^{d}	922^{de}	922^{e}	912^{f}	934^{d}	922^{e}	0.23	0.01	0.01	0.33
CP	90^{e}	82^{f}	104^{d}	106^{d}	97^{d}	90^{e}	99^{d}	1.57	0.01	0.01	0.01
NDF	554^{e}	534^{f}	522^{f}	574^{d}	558^{e}	532^{f}	548^{d}	4.33	0.01	0.01	0.01
ADF	340^{e}	317^{f}	321^{f}	355^{d}	348^{d}	322^{f}	332^{e}	3.33	0.01	0.01	0.01
Lignin	70^{de}	64^{e}	75^{d}	64^{e}	71	66	68	1.75	0.01	0.22	0.11
ME†	9.64^{e}	9.96^{d}	9.88^{d}	9.42^{f}	9.52^{e}	9.90^{d}	9.75^{d}	0.05	0.01	0.01	0.01

Values are expressed as means. Different letters in the same row indicate significant difference ($P<0.05$), YLN=yellow local native, WLN=white local native. †ME, MJ/kg DM (Menke and Steingass, 1988; where ME=14.51- (0.143xADF), where ME= (MJ/kg DM) and ADF= (g/kg DM).

The highest gas production (ml gas/g DM) ($P<0.01$) was for hybrids compared with local natives (Table 2, Fig. 1). There were no differences ($P>0.05$) for fractional lag time and c rate between varieties; the DMd was higher ($P<0.01$) for CLO8001 and WLN compared with H51EA. The RGP was higher for H51EA ($P<0.01$) compared to the rest. ME content was higher ($P<0.01$) for CLO80001>H51EA>YLN>WLN. Regarding the effect of treatment (Fig. 2), there were no differences ($P>0.05$), except for ME, which were higher ($P<0.01$) ENZ and AAC compared with CTR treatment; Corral-Luna *et al*. (2011) found values of ME from 9.62 to 10.46 MJ ME/kg DM in corn silage treated with additives, similar to in the present study. The results indicate that the addition of ENZ reduces the amount of CP, NDF and ADF; this may be due to the impact of enzymes in the inoculum, which may act to degrade the structural carbohydrates (Muck and Bolsen, 1991).

Table 2. *In vitro* gas production (ml gas/g DM) of corn natives and hybrids silage, preserved by the addition of additives

Item	Varieties (*V*)				Treatment (*T*)			SEM	P value		
	YLN	CLO8001	H51EA	WLN	CTR	ENZ	AAC		*V*	*T*	*VxT*
b	282^{e}	319^{d}	316^{d}	297^{e}	306	300	304	8.34	0.01	0.85	0.42
c	0.049	0.050	0.051	0.047	0.047	0.053	0.049	0.01	0.77	0.50	0.97
Lag time	1.36	1.40	1.42	1.34	1.44	1.43	1.28	0.13	0.70	0.98	0.96
DMd,%	63de	65^{d}	59^{e}	63^{d}	61	63	63	0.94	0.01	0.16	0.53
NDFd,%	52	51	51	50	49^{e}	52^{d}	52^{d}	0.59	0.30	0.01	0.01
RGP	340^{e}	362^{e}	414^{d}	341^{e}	367	366	360	8.56	0.01	0.81	0.29

Values are expressed as means of different literals indicate significant difference ($P<0.05$), YLN=local native yellow, white WLN=White local native, CTR=untreated, ENZ=treatment with enzimes (Sill All®), AAC= acetic acid treatment. b=total gas production (ml gas/g DM incubated), c=range of fermentation (h) Lag time (initial fermentation time); DMd%=percentage of DM disappeared; RGP=relative gas production (mL gas/g DMd%); NDFd%= percentage of NDF disappeared.

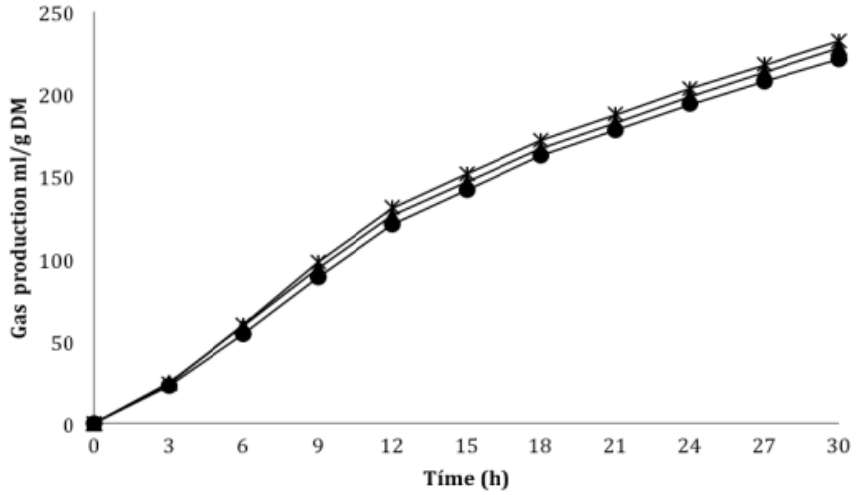

Fig. 1. *In vitro* gas production (ml gas/g DM) of corn silage by treatment (●, control-untreated; ⋆, enzyme (Sill All ®); and ▲, acetic acid).

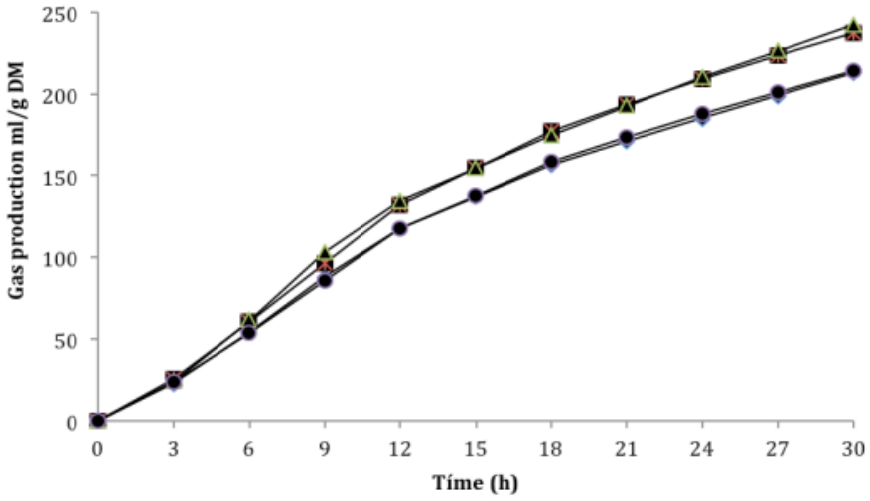

Fig. 2. *In vitro* gas production (ml gas/g DM) of silage corn variety (◆, Yellow local native; ■, CLO80001; ▲, H51EA and ●, White local native).

CONCLUSIONS

Nutritive value and fermentation of corn silage can be improved with treatment of acetic acid or enzyme inoculants. Addition of corn silage with acetic acid or enzymes increased NDF digestibility and ME availability.

ACKNOWLEDGEMENTS

Mr. Ruiz Perez was granted for a CONACyT fellowship during his studies in the University Autonomous-State of Mexico, as a Doctorate Student. This study was supported by the project UAEMex 2750/2009 and Fundacion Produce State of Mexico. We also thank Miss Liz Hopper, LTC University of North Texas for the critical review of the present manuscript.

REFERENCES

AOAC. 1991. *Official Methods of Analysis*, 15th ed.: Association of Official Analytical Chemistry INC. VA, USA.

Colombato, D.F., Mould, M.K., Bhat, R.H. and Phipps Owen, E. 2003. *In vitro* evaluation of fibrolytic enzymes as additives for maize (*Zea mays* L.) silage. II: Effects on rate of acidification, fibre degradation during ensiling and rumen fermentation. *Animal Feed Science and Technology*. 111: 129-143.

Corral-Luna, A., Dominguez-Diaz, D., Rodríguez-Almeida, F.A., Villalobos-Villalobos, G., Ortega-Gutiérrez, J.A. and Muro-Reyes, A. 2011. Composición química y cinética de degradabilidad de ensilaje de maíz convencional y sorgo de nervadura café. *Revista Brasileira de Ciencias Agrícolas*, 6: 181-187.

Filya, I., Sucu, E. and Karabulut, A. 2006. The effect of Lactobacillus buchneri on fermentation, aerobic stability and ruminal degradability of maize silage; *Journal of Applied Microbiology*, 101: 1216-1223.

Getachew, G., Blummel, M., Makkar, H.P.S. and Becker, K. 1998. *In vitro* gas measuring techniques for assessment of nutrition quality of feeds: a review. *Animal Feed Science and Technology,* 72: 261-281.

Gonzalez Ronquillo, M., Fondevilla, M., Barrios, UA. and Newman, Y. 1998. *In vitro* gas production from buffel grass (*Cenchrus ciliaris L*). Fermentation in relation to the cutting interval, the level of nitrogen fertilization and the season of growth. *Animal Feed Science and Technology*, 72: 19-35.

Ivan, S.K., Grant, R.J., Weakley, D. and Beck, J. 2005. Comparison of a corn silage hybrid with high cell-wall content and digestibility with hybrid of lower cell-wall content on performance of Holstein cows. *Journal of Dairy Science*, 88: 244-254.

Johnson, L.M., Harrison, J.H., Davidson, D., Mahanna, W.C. and Shinners, K. 2003. Corn silage management: Effects of hybrid, chop length and mechanical processing on digestion and energy content. *Journal of Dairy Science*, 86: 208-231.

Krishnamoorthy, U., Soller, H. and Menke, K.H. 1991. A comparative study on rumen fermentation or energy supplements *in vitro*. *Journal of Animal Physiology Animal Nutrition,* 65: 28-35.

Kristensen, N.B., Sloth, K.H., HØjberg, O., Spliid, N.H., Jense, C. and ThØgersen, R. 2010. Effects of microbial inoculants on corn silage fermentation, microbial contents, aerobic stability, and milk production under field conditions. *Journal of Dairy Science,* 93: 3764-3774.

Kung, Jr., Chen, J.H., Kreck, E.M. and Knutsen, K. 1993. Effect of microbial inoculants on the nutritive value of the corn silage for lactating dairy cows. *Journal of Dairy Science*, 76: 3763-3770.

Kung, L. and Shaver, R. 2001. Interpretation and use of silage fermentation. Analysis reports. In: *Focus on Forage*. Wisconsin Team Forage. Vol. 3: No. 13.

McDonald, P., Henderson, A.R. and Heron, SJE. 1991. The biochemistry of silage, Chalcombe publications, London UK. 340 pp.

Menke, K.H. and Steingass, H. 1998. Stimulation of the energy fed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research Development*, 28: 7-12.

Muck, R.E. and Bolsen, K.K. 1991. Silage preservation and silage additive products. In: *Field Guide for Hay and Silage Management in North America* (Eds. K.K. Bolsen, J.E. Baylory and M.E. McCullough), Natl. Feed ingred. Assoc. West Des Moins IA, p. 105.

NRC. 2001.Nutrient Requirements of Dairy Cattle. 7th ed. National Academic Press. Washington. D.C.

Ranjit, N.K., Taylor, C.C. and Kung, Jr. L. 2002. Effect of *Lactobacillus buchneri* 40788 on the fermentation, aerobic stability and nutritive value of maize silage. *Grass and Forage Science*, 57: 73-81.

Ruiz, B.O., Castillo, Y., Anchondo, A., Rodriguez, C., Beltran, R. and La O O y Payan, J. 2009. Efecto de las enzimas inoculantes sobre la composición del ensilaje de maíz. *Archivos de Zootecnia*. 58: 163-172.

SAS. 1999. *User's Guide: Statistics version 8*, Statistical Analysis System Institute, Cary, NC. USA.

Servicio de Información Agroalimentaria y Pesquera (on line). http: //www.siap.sagarpa.gob.mx/ (June, 20 2012).

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B. and France, J.A. 1994. Simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology*, 48: 185-197.