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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

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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.

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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.

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