UNIVERSIDAD AUTÓNOMA DEL ESTADO DE MÉXICO
FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA

"Sustainable control of fermentation, methane and carbon dioxide emissions of agricultural calves farming using xylanase and *Saccharomyces cerevisiae*"

ARTICULO ESPECIALIZADO PARA PUBLICAR EN REVISTA INDIZADA

QUE PARA OBTENER EL TÍTULO DE
MÉDICO VETERINARIO ZOOTECNISTA

PRESENTA

HUMBERTO CRUZ GARDUÑO

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TOLUCA, MÉXICO, DICIEMBRE DE 2016.

Sustainable control of fermentation, methane and carbon dioxide emissions of agricultural calves farming using xylanase and *Saccharomyces cerevisiae*

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Abstract

The aim of the present study was to evaluate the potential of supplementing calves diets with exogenous enzymes (xylanase) and yeast (*Saccharomyces cerevisiae*) on the sustainable control of methane (CH_4) and carbon dioxide (CO_2) productions in agricultural calves farming. Three different levels of supplemented diets of xylanase (0, 3 and 6 mg/g of DM), *S. cerevisiae* (SC) (0, 2 and 4 mg/g of DM) and mixture of xylanase and SC (0, 2 µ xylanase + 2 mg *S. cerevisiae*, 6 µ xylanase + 4 mg yeast mg/g of DM) were tested. Asymptotic gas production consistently decreased by each of the additives with the lowest value at the high dose of xylanase + *S. cerevisiae* ($P<0.05$) compared with the control and the low dose of xylanase + *S. cerevisiae*. Methane production was reduced by additives inclusion ($P<0.05$) when compared with the control treatment with no additive. Xylanase + *S. cerevisiae* at all doses increased CO_2 production ($P<0.05$) whereas the high dose had the most significant reduction in gas and methane productions compared with control, xylanase and *S. cerevisiae* additives at different doses. The findings of this study indicate that inclusion of xylanase or SC additives can improve rumen fermentation and reduce greenhouse gases production. The study also established that the mixture of xylanase and *S. cerevisiae* is more efficient in reducing gas and methane emissions for cleaner environmental production conditions in calves farming.

Keywords: Sustainable control, Carbon dioxide, Methane, *Saccharomyces cerevisiae,* Xylanase.

1. Introduction

Ruminant production plays an essential role in the provision of human nutrients and also in the improvement of the world economy. It has been estimated that while the growth of global population will surpass nine billion by the year 2050, food requirements will also increase by 70% compared with the present day needs (FAO, 2009). Similarly, it is expected that the demand and increase in

purchasing power for food from animal sources will rise to 465 and 1.043 million tons for meat and milk products, respectively (FAO, 2006).

However, the major constraints in ruminant farming include: excessive excretion of nutrients, inefficient digestibility and high methane (CH_4) which represent a significant net loss of 2-12% of gross dietary energy (Hristov et al., 2015). The efficient reduction of such energy losses may be potentially used for the production of more meat and milk rather than contributing to greenhouse gas (GHG) production that impacts negatively on climate change (Eckard et al., 2010).

Today, agricultural waste products are one of the largest contributors of anthropogenic sources of three major GHGs: CH_4, carbon dioxide (CO_2), and nitrous oxide (N_2O), with livestock production accounting approximately two-thirds of the direct emissions (Slade et al., 2016), largely from digestion by livestock. Methane has more potent global warming effect of 25-fold higher than CO_2. Methane is the major GHGs emitted from enteric fermentation through the typical digestive process of ruminants production system (Johnson and Johnson, 1995; Hristov et al., 2015) which accounts for approximately 12-17% GHG emission (Beauchemin et al., 2009).

It is essential to know that the production of GHGs from animals and their significant effects on climate changes are a major concern in the world (Martin et al., 2010). Equally, the use of exogenous enzymes and yeast additives in ruminant diets has attracted considerable interest. Many researches have shown that supplementing fiber degrading enzymes in livestock diets will improve the forage quality, increase digestibility, rumen fermentation, and ruminant production (Rojo et al., 2015; Valdes et al., 2015; Morsy et al., 2016), with no effect due to enzyme inclusion in the diet of ruminants (Lewis et al., 1999). The inconsistencies maybe attributed to several factors such as the source of the enzymes (Khattab et al., 2011), doses and activities of the enzyme (Jalilvand et al., 2008), physical properties of the substrate, treatment duration, enzyme application method, diet composition of the animals to which

the enzyme is added (Elghandour et al., 2016a) and level of animal productivity (Beauchemin et al., 2003).

Several studies have reported that supplementation of yeast to ruminants diets improved digestibility of low quality forages (Elghandour et al., 2014; Ahmed et al., 2015; Salem et al., 2016) and altered microbial environment by increasing the number of ruminal microflora which can enhance fiber fraction digestion.

Some researchers have focused on modification of animal diets and the use of additives to combat enteric fermentation and also to reduce rumen GHG productions. This study, therefore, aimed at evaluating the use of feed additives including exogenous enzyme (xylanase), yeast (*Saccharomyces cerevisiae*) and their mixture to control and mitigate the gas emissions from the agriculture calf farms.

2. Materials and methods

2.1. In vitro incubations and treatments

Rumen inoculum was collected by stomach tube from 40 weaned Holstein calves (40 to 55 kg body weight) before morning feeding, divided into 4 groups (n=10) and fed a basal diet with no additive (Control), or daily supplemented with 5 mL of xylanase (Dyadic PLUS; Dyadic international, Inc, Jupiter, FL, USA), or 4 g of *S. cerevisiae*, with a minimum guaranteed concentration of live yeast cells of 1.5 x 10^{10} CFU of *S. cerevisiae*/g of product (Procreatin 7, Safmix, Toluca, Mexico) or their mixture (2.5 mL xylanase + 2 g yeast) for 60 days of age. Calves were fed *ad libitum* a total mixed ration of a commercial concentrate (Ultra Malta Clayton®, Toluca, Mexico) formulated to meet their nutrient requirements (NRC 1985) with free access to fresh water. The diet contained (per kg DM) of 200 g crude protein, 230 g neutral detergent fiber (NDF), 50.3 g acid detergent fiber (ADF) and 35.6 g ether extract. The treatments which were tested against control (no additives) were as follows: xylanase (at 3 and 6 µL/g DM), yeast (at 2 and 4 mg/g DM) and their mixture (at 3µ xylanase + 2 mg yeast, and 6 µ

xylanase + 4 mg yeast). The diet fed to calves was used as the substrate for the *in vitro* incubation. The product of xylanase contained: 34,000 to 41,000 U of xylanase/mL, 12,000 to 15,000 units of beta-glucanase/mL, and 45,000 to 55,000 U of cellulase/mL.

Immediately after collection, the rumen contents obtained from the donor calves were flushed with CO_2, mixed and strained through four layers of cheesecloth into a flask with O_2 free headspace. Filtered rumen fluid was immediately transported to the laboratory where it was mixed in a 1:4 (v/v) proportion with the buffer solution described by Goering and Van Soest (1970), with no trypticase added. Diluted rumen fluid (50 mL containing 10 mL of rumen liquor) was added to each incubation bottle containing 0.5 g of substrate, which had been previously weighed out and additive solutions dispensed.

Three incubation runs were performed in three different weeks. Bottles were inoculated within each incubation run, with three bottles as blanks (i.e., rumen fluid only, with no substrate or additive). After filling all bottles, they were flushed with CO_2 and immediately closed with rubber stoppers, shaken and placed in a water bath at 39°C. The volume of gas produced was recorded at 2, 4, 6, 8, 10, 12, 14, 16, 18, 24, 36, 48 and 70 h of incubation using a pressure transducer (Extech Instruments, Waltham, USA) following the technique of Theodorou et al. (1994). At the same incubation times, CH_4 and CO_2 concentrations in the headspace of the bottles were measured using a diffusion based gas detector (Gas Analyzer CROWCON Model Tetra3, Abingdon, UK).

After sampling the supernatant for pH determination, the contents of each bottle were filtered under vacuum through sintered glass crucibles (coarse porosity no. 1, pore size 100 to 160 μm; Pyrex, Stone, UK). The incubation residues were dried then at 70 °C overnight to estimate apparent DM disappearance.

2.2. Chemical analyses

Samples of the incubated substrate were analyzed for DM (#934.01), ash (#942.05), N (#954.01) and ether extract (#920.39) using AOAC (1997) official methods. The NDF (Van Soest et al. 1991) and ADF (AOAC #973.18) contents were determined using an ANKOM^{200} Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA). The NDF analysis was done with sodium sulfite, and with α-amylase. Both NDF and ADF were expressed without residual ash.

2.3. Calculations and Statistical analyses.

To estimate kinetic parameters of gas production (GP), gas volumes recorded (mL/g DM) were fitted using the NLIN procedure of SAS (2002) according to France et al. (2000) model as:

$$y = b \times [1 - e^{-c\,(t-Lag)}]$$

where y is the volume of GP at time t (h); b is the asymptotic GP (mL/g DM); c is the fractional rate of fermentation (/h), and Lag (h) is the discrete lag time prior to any gas being released.

The experimental design for the *in vitro* ruminal GP and fermentation parameters analysis was a completely randomized design considering, as fixed factors, additive type and additive doses in the linear model (Steel and Torrie 1980). Data of each of the three runs within the same sample were averaged prior to statistical analysis. Mean values of each individual extract within each species (three samples of each) were used as the experimental unit. Linear and quadratic polynomial contrasts were used to examine responses to increasing addition of additives doses. Multiple comparisons of means were performed using the Tukey's test.

3. Results

Table 1 shows the *in vitro* gas, CH_4 and CO_2 kinetics as affected by the addition of enzymes, yeast and their mixture. There was observable linear decrease in asymptotic GP at all doses of xylanase, yeast and xylanase + yeast supplemented diets compared with the control treatment (with no additive). The supplementation of xylanase and *S. cerevisiae* to the diets of the calves had no significant effects ($P > 0.05$) on the asymptotic GP at all tested doses, while the supplementation of a mixture of xylanase + yeast at high dose affected it ($P < 0.05$) compared with the control. The rate of GP showed positive effect ($P< 0.05$) on all the doses of xylanase addition and no significant effect ($P > 0.05$) with the addition of yeast and xylanase + yeast additives when compared with the control. In addition, there was an increase in the lag time of GP at each dose of xylanase, yeast and xylanase + yeast additives, and a significant effect ($P < 0.05$) on all the doses of xylanase and yeast additives compared with the control.

Xylanase, yeast and xylanase + yeast in all doses except for 0 mg dose of xylanase + yeast additive affected ($P < 0.05$) CH_4 production when compared with the control treatment. Also the mean production of CH_4 from the xylanase, yeast and xylanase + yeast differed ($P<0.05$) with the control. The lowest asymptotic CH_4 production was observed at the high dose of xylanase + yeast which is lower ($P<0.05$) than that of the control treatment. No effect ($P> 0.05$) was observed in all the doses in the rate of CH_4 production except at 3 mL xylanase/g DM that differed ($P< 0.05$) compared with the control. Xylanase had effect on the mean rate of CH_4 production and no observable effects ($P> 0.05$) was observed with the addition of yeast and xylanase + yeast additives. In addition, there was no significant increase ($P> 0.05$) in the lag time of CH_4 production at all doses when compared with the control treatment.

The mean asymptotic CO_2 production was higher ($P<0.05$) when yeast and xylanase + yeast additive addition than the control (without additive). The highest asymptotic CO_2 production was recorded for the treatment containing 2 mg yeast/g DM and high dose of xylanase + yeast, and greater ($P< 0.05$) than the control but a decrease below the control was observed at 6 mL xylanase/g DM and 0

mg yeast /g DM. The mean rate of GP differed ($P<0.05$) only with the addition of xylanase and not with yeast and xylanase + yeast additives when compared with the control. All the values of the lag time of CO_2 production ranged from the lowest scale of 8.18 mL/g DM in 2 mg yeast to the highest gauge of 10.88 mL/g DM in the high dose of xylanase + yeast. There was no significant effect ($P> 0.05$) on mean lag time of CO_2 production for xylanase, yeast additives and the control, but a significant effect was observed for xylanase + yeast compared with the control treatment.

Table 2 shows the *in vitro* CH_4 and CO_2 productions at 6, 24 and 48 h after incubation as affected by the addition of enzymes, yeast and a mixture of both in rumen liquor of calves fed on diet supplemented with the same three additives for 60 days of age. At 6, 24 and 48 h of incubation, xylanase, yeast and xylanase + yeast did affect CH_4 production (mL/g incubated DM) compared with the control treatment, and there was a reduced effect ($P< 0.05$) on CH_4 production at 24 and 48 h of incubation. However, the mixture of xylanase and yeast had no effect on the CH_4 production at 24 and 48 h compared with their respective controls. Moreover, CH_4 production (mL/g degraded DM) was decreased at the high dose of xylanase + yeast at 24 and 48 h of incubation. There was an observable reduction in the CH_4 production (mL/g degraded DM) in xylanase, yeast and mixture of xylanase + yeast compared with the control. The proportional CH_4 production at 6, 24 and 48 h of incubation was reduced slightly but registered no effect ($P> 0.05$) compared with the control treatment, while addition of additives resulted in a decreased proportional CH_4 production. On the other hand, addition of xylanase, yeast and xylanase + yeast increased the production of CO_2 production (mL/g incubated DM) and mL/g degraded DM but had no effect ($P>0.05$) on the proportional CO_2 production when compared with the control treatment.

Fig. 1 shows the *in vitro* rumen gas, CH_4 and CO_2 productions (mL/g incubated DM) of calf's diet supplemented with xylanase, yeast and xylanase + yeast. Xylanase, yeast and xylanase + yeast had

a reduced effect ($P<0.05$) on asymptotic GP which was dose dependent. The different doses of the additives decreased ($P<0.05$) CH_4 production (mL/g incubated DM) and increased CO_2 production.

4. Discussion

Agricultural wastes are important sources of GHG emissions and globally it is being estimated to rise about 8.2 billion tons of CO_2 equivalents by 2030, if adequate mitigation technique is not properly implemented (Slade et al., 2016). Apart from the impacts of GHG, enteric CH_4 emission contributes to a loss of net feed energy that cannot be used in ruminant animals for production purposes (Johnson and Johnson, 1995). Because of these challenges intensive research efforts are recently directed towards ruminant animals CH_4 mitigation (Elghandour et al., 2016b,c). The use of *in vitro* GP technique is a powerful, simple and sensitive screening method for evaluating substrate fermentation or degradation and for monitoring the efficacy of feed additives (Elghandour et al., 2015; Vallejo et al., 2016) and GHG production (Elghandour et al., 2016a,b).

In this study, asymptotic GP decreased linearly as the doses of xylanase, yeast and their mixture increased showing that the higher doses of these additives have potential influence to modify rumen GP kinetics. The addition of enzyme at all doses had no significant effects on the asymptotic GP. This finding is similar to the report of Jalilvand et al. (2008) who observed that the addition of enzyme additives to forage had negligible effects on GP kinetics and opined that the effects of enzyme addition depend on the fiber content, structural polysaccharide compositions of the substrate and difference in enzyme composition. Enzyme addition significantly affected the rate of GP, which contrasts previous reports on other enzyme preparations and types (Jalilvand et al., 2008). Many recent studies including *in vivo* (Morsy et al., 2016; Rojo et al., 2015) and *in vitro* (Elghandour et al., 2016a) experiments showed that supplementation of ruminant diets with exogenous enzymes can improve feed utilization, digestion of DM, and animal performance by improving DM degradations (Alsersy et al., 2015).

Addition of yeast additives decreased asymptotic GP compared with the enzyme additives at all doses but showed no significant effects when compared with the control treatment. Several reports have shown that supplementation of *S. cerevisiae* to diets of ruminants improved feed utilization (Hassan et al., 2016). In contrast, Corona et al., (1999) reported that supplementation of *S. cerevisiae* to cow diets did not affect digestibility of DM, hemicellulose and starch. Yeast additives had no significant effects on rate of GP but there was a slight increase in rate of GP compared with the control. This result is in contrast to the work of Elghandour et al. (2014) and Rodriguez et al. (2015) who reported a decreased rate of GP in response to yeast additives. The differences may be due to the composition and incubation of the substrates (Elghandour et al., 2014). Addition of a mixture of enzymes and yeast at a high dose significantly reduced the asymptotic GP compared with other treatments.

At 6, 24 and 48 h of incubation, addition of additives at all doses did not affect CH_4 production compared with the control treatment, and there was observable significant reduction effect at 24 and 48 h of incubation on CH_4 production. However, a mixture of xylanase and yeast had no effect on the CH_4 production at 24 and 48 h compared with the control treatment. Xylanase, yeast and their mixture at all doses except that of 0 mg dose of xylanase + yeast decreased CH_4 production when compared with the control treatment. This pronounced decrease in CH_4 suggests that the use of xylanase, yeast or its mixture as additives in ruminant diets may serve as efficient methods to reduce CH_4 emission from ruminant production. Several researches have reported a reduction of CH_4 production with yeast supplementation. For instance, 20 and 58% reduction in CH_4 production have been reported by Lynch and Martin, (2002) and Newbold and Rode, (2006), respectively with the addition of yeast in ruminant diets. Besides, Polyorach et al. (2014) noted a significant decrease in *in vitro* CH_4 production with supplementation of *S. cerevisiae*, which supports our findings.

Of the several studies which have evaluated the effects of exogenous enzymes on CH_4 emission in the rumen, few reported an absolute increase in production of CH4 with addition of exogenous enzymes supplementation to the ruminant diets (Dong et al., 1999; Beauchemin et al., 2009). Dong et al. (1999) reported 43% increase on CH_4 production when cellulase and xylanase were used as supplements with hay in RUSITEC system. Another researcher reported that there was no effect of enzymes supplement in CH_4 production in continuous culture system (Colombatto et al., 2003) and in steers fed with barley silage-based diets supplemented with different feed additives including exogenous enzymes (McGinn et al., 2004). In contrast, some other researchers reported that the addition of enzymes at certain doses reduced CH_4 production in equine diets (Salem et al., 2015; Kholif et al., 2016). In the present study, addition of enzymes at all doses decreased CH_4 production. This may be due to the possible stimulation of reductive acetogens in the rumen that alters H_2 metabolism and its utilization by methanogens in a manner that will reduce CH_4 formation and emissions (Stewart et al., 1997). The reduction in CH_4 by the addition of a combination of xylanase and yeast at high dose is a positive impact on rumen fermentation, although the observed pronounced decrease in CH_4 production at high dose of the added additive was accompanied by a slight decrease at high dose of asymptotic GP, indicating a direct inhibitory effect of rumen fermentation kinetics.

Many gases consisting of mainly CH_4 CO_2 and H_2 are produced during ruminal fermentation process within the rumen. In this study, addition of additives at all doses slightly increased CO_2 production at 6, 24 and 48 h of incubation. At 6 h of incubation, there was no CO_2 production in all the additives as well as in the control. Decreased CO_2 production below the control treatment was observed at 6 mL xylanase/g DM and 0 mg yeast/g DM. This reduction in CO_2 production and decrease in rate of CO_2 may be due to increased cell wall content that can reduce the microbial activities. Elghandour et al. (2016c) reported a decreased CO_2 production when corn grain was replaced by soybean hulls and they attributed the decrease to increased fibers and decreased non-structural carbohydrates when corn

grains were replaced with soybean hulls. To the best of our knowledge, there is little information on the effects of supplementing diets of ruminants with enzymes and yeast additives on CO_2 production which makes it difficult to compare the present results. The asymptotic CO_2 production recorded the highest values for 2 mg yeast/g DM (90.2 mL/g DM) and (96.9 and 79.6 mL/g DM) for 0 and high dose of xylanase + yeast of which three of them were significantly greater than the control treatment (37.5 mL/g DM).

5. Conclusion

Supplementation of ruminant's diets with xylanase, yeast and their mixture at different doses for 60 days of age changed the pattern of ruminal production of GP, CH_4 and CO_2. Addition of a mixture of enzymes and yeast at a high dose significantly reduced the asymptotic GP compared with other treatments with dose-dependent manners. Addition of additives in the diets of ruminants at all doses had significant reduction effect on CH_4 production. The pronounced decrease in CH_4 production by all the additives supplemented to ruminant diets in this study may serve as one of the most effective methods of sustainable reduction of CH_4 emission that can provide cleaner environmental conditions for ruminant production.

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Table 1.
In vitro gas, methane (CH_4) and carbon dioxide (CO_2) kinetics as affected by addition of xylanase, yeast and mixture of both in rumen liquor of calves fed on diet supplemented with xylanase and/or yeast for 60 days of age

Rumen liquor from calves fed on:	Additive:	Dose (mg/g DM)	Gas production (mL/0.5 g DM)[1]			CH_4 production (mL/g DM)[2]			CO_2 production (mL/g DM)[3]		
			b	*c*	*Lag*	*b*	*c*	*Lag*	*b*	*c*	*Lag*
Control	No additive	0	382.9^{Aa}	0.058^{Cc}	2.69^{Ce}	108.2^{Aa}	0.044^{Bbc}	7.15^{ab}	37.5Ced	0.041Bc	8.48Bc
Enzyme	Xylanase	0µL	374.6^{a}	0.141^{a}	5.28^{abc}	67.1^{bc}	0.093^{ab}	8.93^{a}	57.6^{c}	0.147^{ab}	10.17^{ab}
		3µL	348.2^{a}	0.148^{a}	5.28^{abc}	60.4^{c}	0.102^{a}	8.30^{ab}	49.9^{cd}	0.162^{a}	9.78^{abc}
		6µL	302.8^{ab}	0.128^{ab}	5.43^{abc}	64.2^{c}	0.070^{abc}	6.87^{ab}	35.2^{ed}	0.060^{c}	8.39^{c}
		Mean	352.1^{AB}	0.119^{A}	4.67^{A}	75.0^{B}	0.077^{A}	7.81	45.1^{BC}	0.103^{A}	9.21^{AB}
Yeast	Yeast	0mg	330.6^{ab}	0.000^{bc}	6.59^{a}	63.2^{c}	0.049^{bc}	8.53^{ab}	30.6^{e}	0.074^{c}	8.74^{bc}
		2mg	329.1^{ab}	0.105^{abc}	5.58^{ab}	69.3^{bc}	0.062^{abc}	8.01^{ab}	90.2^{ab}	0.087^{bc}	8.18^{c}
		4mg	294.9^{ab}	0.108^{abc}	6.02^{a}	67.0^{bc}	0.050^{bc}	9.03^{a}	46.6^{cde}	0.043^{c}	8.50^{bc}
		Mean	334.4^{AB}	0.0862^{BC}	5.22^{A}	76.9^{B}	0.051^{B}	8.18	51.2^{B}	0.061^{B}	8.48^{B}
Enzyme + Yeast	Xylanase + Yeast	0	295.4^{ab}	0.09967^{abc}	3.78^{de}	104.8^{ab}	0.042^{c}	7.30^{ab}	96.6^{a}	0.041^{c}	10.49^{a}
		3µENZ+2mg yeast	296.5^{ab}	0.10533^{abc}	4.07^{cde}	74.3^{abc}	0.050^{bc}	6.40^{b}	57.6^{c}	0.064^{c}	9.54^{abc}
		6µENZ+4mg yeast	240.4^{b}	0.11133^{abc}	4.37^{bcd}	55.2^{c}	0.055^{abc}	7.73^{ab}	79.6^{b}	0.049^{c}	10.88^{a}
		Mean	303.8^{B}	0.09367^{AB}	3.73^{B}	85.6^{B}	0.048^{B}	7.15	67.8^{A}	0.049^{B}	9.85^{A}
SEM[4]			20.56	0.01186	0.291	7.45	0.0091	0.434	3.17	0.014	0.327
P value											
Additive			0.138	0.033	<0.001	0.306	0.022	0.579	<0.001	0.008	0.008
Rumen Liquor			0.007	<0.001	<0.001	<0.001	0.038	0.004	<0.001	0.001	<0.001
Doses			0.031	0.255	0.133	0.024	0.551	0.159	<0.001	0.002	0.154
Additive×rumen liquor			0.175	0.027	<0.001	0.017	0.025	0.168	<0.001	0.003	0.030
Additive×dose			0.798	0.468	0.754	0.015	0.109	0.007	<0.001	0.004	0.001

[1]*b* is the asymptotic gas production (mL/0.5 g DM); *c* is the rate of gas production (/h); *Lag* is the initial delay before gas production begins (h).

[2]*b* is the asymptotic methane production (mL/g DM); *c* is the rate of methane production (/h); *Lag* is the initial delay before methane production begins (h).

[3]*b* is the asymptotic carbon dioxide production (mL/g DM); *c* is the rate of carbon dioxide production (/h); *Lag* is the initial delay before carbon dioxide production begins (h).

[4]SEM standard error of the mean.

Means of doses in the same column with different small letter superscripts differ ($P<0.05$).
Means of additives in the same column with different capital letter superscripts differ ($P<0.05$).

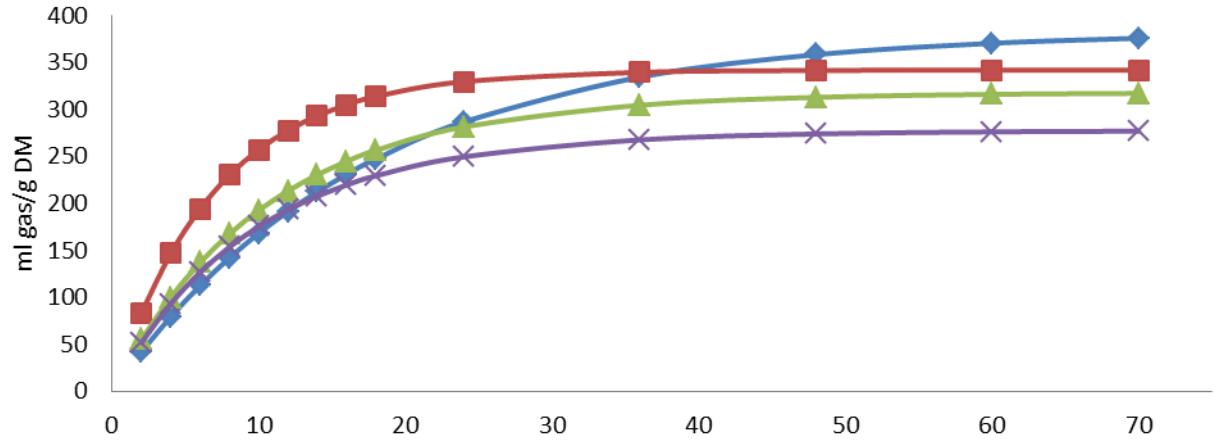
Table 2.

In vitro methane (CH_4) and carbon dioxide (CO_2) production at 6, 24 and 48 h after incubation as affected by addition of xylanase, yeast and mixture of both in rumen liquor of calves fed on diet supplemented with xylanase and/or yeast for 60 days of age

Rumen liquor from calves fed on:	Additive:	Dose (mg/g DM)	DMD	CH_4 production									CO_2 production								
				mL/g incubated DM			mL/g degraded DM			Proportional production		CH_4	mL/g incubated DM			mL/g DM degraded			Proportional production		CO_2
				6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Control	No additive	0	693.3	25.0	70.2 Aa	94.7 Aa	2.47	84.9	127.7 Aa	1.53 Aa	20.6 A	24.8 A	8.2 Bb	23.5 d	32.1 e	0	34.2	64.7	0	8.3	12.7
Enzyme	Xylanase	0µL	656.3	28.8	59.9 ab	66.3 bc	2.56	72.1	82.4 b	0.79 b	13.1	14.5	33.6 b	55.5 b	57.5 c	0	63.8	69.7	0	11.7	12.3
		3µL	644.3	27.5	55.0 ab	59.9 c	2.22	78.4	91.2 b	0.70 b	14.9	16.9	30.9 bc	48.8 bc	49.9 d	0	71.9	77.5	0	13.7	14.3
		6µL	642.3	21.6	48.6 ab	58.6	2.59	62.5	80.7 b	1.00 b	14.0	17.4	10.5	26.4	32.8	0	52.6	67.9	0	11.7	14.3
		Mean	659.1	25.7	58.5 AB	69.9	2.46	74.5	95.55 B	1.00	15.6 AB	18.4 B	20.8 A	38.5 A	43.1	0	55.6	69.9	0	11.3	13.4
Yeast	Yeast	0mg	664.7	16.2	42.9 b	55.9	0.85	53.0	78.8 b	0.51	12.9	16.3	10.8	25.0	29.5	0	36.7	51.4	0	9.0	10.7
		2mg	671.0	21.5	53.6 ab	65.7 bc	1.41	60.3	90.2 b	0.62	13.6	18.8	31.9	65.3	77.3	0	115.4	136.6	0	25.3	27.6
		4mg	625.1	15.9	42.1 b	56.3	1.42	58.7	83.8 b	0.58	12.7	17.1	10.5	29.9 d	40.5 e	0	63.6	80.5	0	13.0	15.3
		Mean	663.5	19.7	52.2 B	68.2	1.54	64.2	95.1	0.81	14.9 B	19.3 B	15.4 AB	35.9 A	44.9	0	62.5	83.3	0	13.9	16.6
Mixture	Xylanase + Yeast	0	681.3	23.3	66.5 ab	90.8 b	2.48	76.6	101.8 ab	1.28 b	19.8	23.6	21.0 b	60.3 b	82.9	0	52.4	74.4	0	12.7	16.0
		3µENZ+2 mg yeast	673.5	18.8	50.4	65.9 bc	2.20	58.3	77.0 b	1.09 b	14.9	18.0	17.7 b	43.7 cd	53.9 d	0	39.3	49.3	0	10.0	11.7
		6µENZ+4 mg yeast	677.6	15.5	40.4 b	51.2	1.80	52.9	67.6 b	1.13 b	16.6	19.3	20.1 b	54.7 b	71.8 b	0	49.7	68.3	0	15.0	19.0
		Mean	681.4	20.7	56.9 AB	75.6	2.24	68.2	93.5	1.26 AB	18.0 AB	21.4 AB	16.8 AB	45.6 A	60.2 A	0	43.9	64.2	0	11.5	14.8
SEM			23.81	2.87	5.28	6.10	0.400	9.53	10.38	0.175	1.96	2.05	3.37	4.07	3.045	0	17.77	23.63	0	3.42	4.33

P value																			
Additive	0.433	0.065	0.170	0.219	0.128	0.439	0.784	0.208	0.607	0.632	0.054	<0.001	<0.001	1.000	0.775	0.937	1.000	0.962	0.962
Rumen Liquor	0.040	0.347	0.004	<0.001	0.134	0.032	<0.001	<0.001	0.003	0.001	<0.001	<0.001	<0.001	1.000	0.257	0.983	1.000	0.330	0.926
Doses	0.422	0.083	0.013	0.008	0.712	0.496	0.476	0.872	0.936	0.840	<0.001	<0.001	<0.001	1.000	0.062	0.178	1.000	0.031	0.092
Additive×rumen liquor	0.431	0.111	0.090	0.025	0.076	0.434	0.499	0.107	0.158	0.081	0.008	<0.001	<0.001	1.000	0.749	0.876	1.000	0.859	0.822
Additive×dose	0.228	0.835	0.361	0.048	0.575	0.443	0.202	0.566	0.245	0.121	0.003	0.001	<0.001	1.000	0.694	0.753	1.000	0.589	0.661

Means of doses in the same column with different small letter superscripts differ (P<0.05).
Means of additives in the same column with different capital letter superscripts differ (P<0.05).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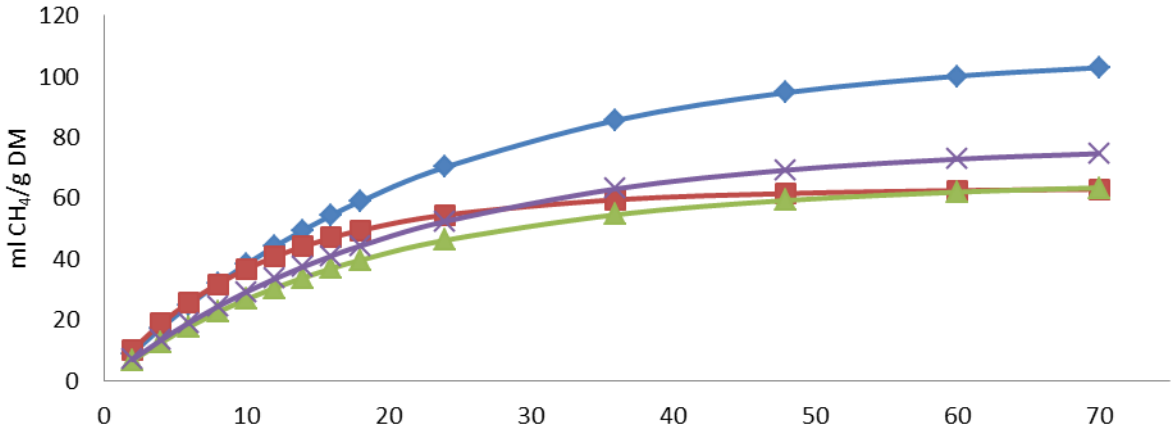
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70


ml CH_4/g DM
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100
80
60
40
20
0
0
10
20
30
40
50
60
70

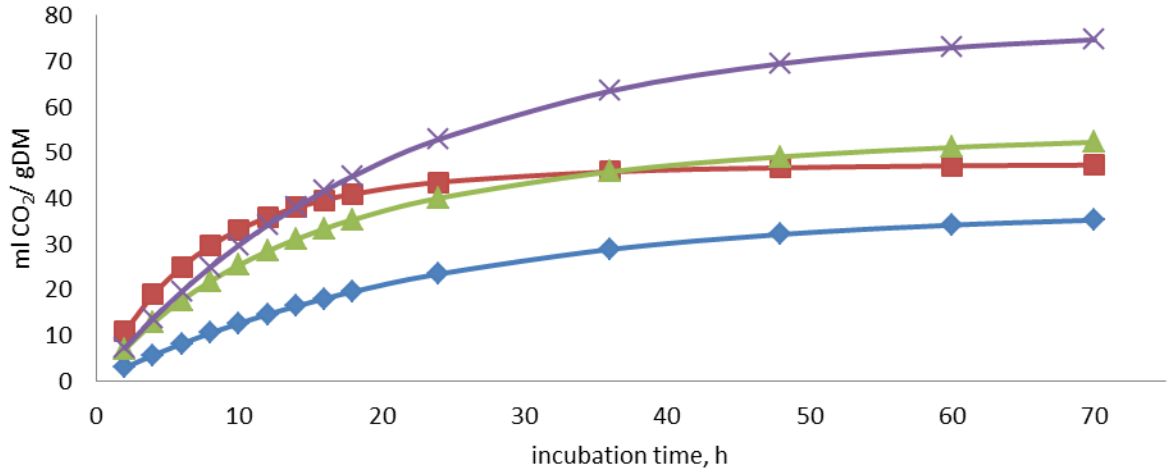
ml CO_2/ gDM
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
incubation time, h

Fig 1.
In vitro rumen gas, CH_4 and CO_2 productions (mL/g incubated DM) of calf's diet supplemented with: control (no additive (-♦-), Xylanase (-■-), *Saccharomyces cerevisiae* (-▲-), and their mixture (-X-) and incubated with rumen inoculum from calves fed on diet supplemented with xylanase and/or yeast for 60 days of age.