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Methane production from four forages at three maturity stages in a ruminal *in vitro* system¤

Producción de metano de cuatro forrajes en tres estados de madurez en un sistema ruminal in vitro

Produção de metano de quatro forragens em três estágios de maturidade em um sistema ruminal in vitro

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Abstract

Background: Forage characteristics can modify *in vitro* methane production. There is little information about *in vitro* methane production of legumes and grasses at different maturity stages in tropical highland grazing systems. **Objective:** To evaluate the effect of species and forage maturity on *in vitro* methane production. **Methods:** Four forage species grown in tropical highlands of Colombia, two grasses: Kikuyu (*Cenchrus clandestinus,* previously named *Pennisetum clandestinum*) and ryegrass (*Lolium perenne var.* Samsum), and two legumes: Lotus (*Lotus uliginosus var.* Maku) and red clover (*Trifolium pratense*) were harvested in two paddocks at three maturity stages (young, intermediate, and mature). *In vitro* 48 h gas production was measured and methane proportion in gas was quantified by gas chromatography. Data were analysed as a randomized complete block (paddocks) design with a factorial arrangement 4×3 (4 species × 3 maturity stages) using the GLM procedure of SAS®. **Results:** Lotus produced less methane ($p<0.01$) than ryegrass, clover, and kikuyu (35.5 vs 64.7, 55.7 or 51.4 mL/g degraded organic matter, respectively). Younger forages produced less methane than intermediate and mature forages (42.8 vs 56.3 and 56.4 mL/g degraded organic matter, respectively). Cellulose concentration and organic matter degradability explained 67% ($p<0.01$) of methane production. **Conclusion:** Forage composition, presence of condensed tannins, and changes in fermentation patterns may explain the differences observed in *in vitro* methane production among species and maturity stages.

Keywords*:* *grassland systems, kikuyu, lotus, methanogenesis, red clover, ryegrass.*

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Resumen

Antecedentes: Las características de los forrajes pueden afectar la producción de metano. Hay poca información sobre la producción de metano de leguminosas y gramíneas en diferentes estados de madurez en los sistemas pastoriles de trópico alto. **Objetivo:** Evaluar el efecto de la especie y la madurez de los forrajes sobre la producción de metano *in vitro*. **Métodos:** Cuatro especies forrajeras de trópico alto colombiano, dos gramíneas: Kikuyo (*Cenchrus clandestinus,* anteriormente llamado *Pennisetum clandestinum)* y ryegrass (*Lolium perenne var.* Samsum) y dos leguminosas: Lotus (*Lotus uliginosus var.* Maku) y trébol rojo (*Trifolium pratense*), fueron cosechadas de dos parcelas en tres estados de madurez (joven, intermedio y maduro). Se midió la producción de gas *in vitro* a las 48 h y la proporción de metano en el gas por cromatografía de gases. Los datos se analizaron empleando un diseño de bloques completos al azar (parcelas) con un arreglo factorial 4×3 (4 especies × 3 estados de madurez) mediante el procedimiento GLM de SAS®. **Resultados:** El lotus produjo menos metano (p<0,01) que el ryegrass, trébol o kikuyo (35,5 vs 64,7, 55,7 o 51,4 mL/g materia orgánica degradada, respectivamente). Los forrajes jóvenes produjeron menos metano que aquellos de edad intermedia y madura (42,8 vs 56,3 y 56,4 mL/g materia orgánica degradada, respectivamente). La concentración de celulosa y la digestibilidad de la materia orgánica explicaron el 67% (p<0,01) de la producción de metano. **Conclusión:** La composición de la pastura, la concentración de taninos condensados y los cambios en los patrones de fermentación pueden explicar las diferencias en la producción de metano *in vitro* según la especie y madurez del forraje.

Palabras claves: *kikuyo, lótus, metanogénesis, ryegrass, sistemas pastoriles, trébol rojo.*

Resumo

Antecedentes: As caraterísticas das forragens podem modificar a produção de metano. Existe pouca informação sobre a produção de metano de leguminosas e gramíneas em diferentes fases de maturidade nos sistemas de pastoreio de trópico alto. **Objetivo:** Avaliar o efeito da espécie e a maturidade das forragens sobre a produção de metano *in vitro*. **Métodos:** Quatro espécies de forragens de trópico alto colombiano, duas gramíneas: Capim quicuio (*Cenchrus clandestinus),* antigamente chamado *Pennisetum clandestinum*) e azevém (*Lolium perenne var.* Samsum) e duas leguminosas: Trevina (*Lotus uliginosus var.* Maku) y trevo (*Trifolium pratense*), foram colhidas de duas parcelas diferentes em três fases diferentes de maturidade (jovem, intermedia e madura). Foi avaliada a produção de gás *in vitro* às 48 h e a proporção de metano foi determinada por cromatografia de gases. Os dados foram analisados com um delineamento em blocos ao acaso (parcelas) com um arranjo fatorial 4×3 (4 espécies × 3 fases de maturidade) utilizando o procedimento GLM de SAS®. **Resultados:** Trevina produz menos metano (p<0,01) que azevém, trevo o capim quicuio (35,5 vs 64,7, 55,7 ou 51,4 mL/g matéria orgânica degradada, respetivamente). Forragens jovens produzem menos metano que forragens com idade intermedia e matura (42,8 vs 56,3 y 56,4 mL/g matéria orgânica degradada, respetivamente). A concentração de celulosa e a digestibilidade da matéria orgânica explicaram o 67% (p<0,01) da produção de metano. **Conclusão:** A composição da pastagem, a concentração de taninos condensados e as mudanças nos padrões de fermentação podem explicar as diferencias na produção de metano *in vitro* entre espécies e maturidade de forragens.

Palavras-chave: *kikuyo, metanogênese, ryegrass, sistemas de pastorais, trevina, trevo.*

Introduction

Methane, a major greenhouse gas (GHG) has 28 times more heating power than carbon dioxide and average persistency of 12.4 years in the atmosphere (IPCC, 2014). Enteric methane (CH_4) production accounts for energy losses between 5 and 7% of the gross energy consumed by ruminants (Johnson and Johnson, 1995). Strategies to mitigate the CH_4 generated by ruminants would help decrease GHG emissions by the livestock sector (Niggli *et al.*, 2009).

For a significant proportion of ruminants, pastures are the main source of feed and their characteristics and management can modify CH_4 emissions (Johnson and Johnson, 1995; Lovett *et al.*, 2005; Vargas *et al.*, 2013). In Colombia, kikuyu (*C. clandestinus*), ryegrass (*L. perenne*) and clover (*T. pretense*) are the main species used in highland dairy cattle production systems. The literature is contradictory regarding the effect of forage maturity on CH_4 emissions. Purcell *et al.* (2011) and Navarro-Villa *et al.* (2011) suggested that maturity of *L. perenne* is positively related to methane emissions. However, Purcell *et al.* (2012) reported a decrease in CH_4 emissions for the same grass, when forage maturity increased. We did not find reports assessing the effect of maturity of legumes on *in vitro* CH_4 emissions. Therefore, the objective of this study was to evaluate the effect of three maturity stages of two grasses (*C. clandestinus* and *L. perenne*) and two legumes (*L. uliginosus* and *T. pratense*) on *in vitro* CH_4 production.

Materials and methods

Forage species

Two grasses, perennial ryegrass (*Lolium perenne* var. Samson) and kikuyu (*Cenchrus clandestinus*, previously named *Pennisetum clandestinum*), and two legumes, red clover (*Trifolium pratense*) and big lotus (*Lotus uliginosus* var. Maku) were harvested from two paddocks of grass and legume species during the rainy season in a highland region of Colombia (4° 40' 89" N, 74° 13' 13" W; at 2,540 m.a.s.l.) at three stages of maturity (young, intermediate, and mature), according to the neutral detergent fiber (NDF) concentration (legumes <30, 30 to 34, and >34%; and grasses <40, 40 to 55, and >55% for low, medium and high, respectively). Forages were harvested at 10 cm above soil surface -simulating animal behavior- at 15, 35, or 70 d of regrowth for ryegrass and kikuyu; and at 25, 45, or 90 d of regrowth for clover and lotus (young, intermediate, and mature stages, respectively). The forages were frozen at -20 °C, lyophilized (Alpha 1-4LDplus, Martin Christ®, Christ, Osterode, Germany) at a temperature of -56 °C and a pressure of 0.0035 psi, and ground in a mill (Romer series II, Romer®, Romer Labs, Getzersdorf, Austria) using a 1-mm sieve.

In vitro incubation

Forage samples (lotus, clover, kikuyu, or ryegrass) from each paddock at three maturity stages (young, intermediate or mature) and a blank (without forage) were incubated in triplicate for 48 h in an *in vitro* ruminal system, according to the procedure by Pell and Scofield (1993), adapted by Parra and Avila (2010). Ruminal fluid was obtained from an overnight-fasted bovine fitted with a ruminal cannula and grazing on kikuyu pasture. The fluid was filtered through four layers of gauze, and gassed with CO_2. Three samples (0.1 g) of each forage were placed in 60 mL bottles. Then, 8 mL of a buffer (pH 6.5; Goering and Van Soest, 1970), and 2 mL of ruminal fluid were added to each bottle, gassed with CO_2 and incubated at 39 °C (Inkubator 1000/Titramax1000, Heidolph®, Heidolph Instruments, Schwabach, Franconia, Germany). The bottles were closed with butyl rubber stoppers and sealed with staples.

Gas production was quantified at 0, 2, 4, 8, 12, 18, 24, and 48 h using a manual transducer (Digital Test Gauge, Ashcroft®, Ashcroft Inc., Stratford, CT, USA) which measures the gas volume according to bottle pressure (Theodorou *et al.,* 1994). Total gas production was determined by adding up the partial gas yields at each sampling time. A sample of gas for each sampling time was placed in vacutainers for subsequent determination of CH_4 concentration. At the end of the fermentation period (48 h), pH was determined using a potentiometer (Thermo Scientific Orion 3 start, Thermo Fisher Scientific®, Thermo Fisher Scientific Inc., Madison, USA). Subsequently, a sample of the supernatant (2 mL) was acidified with 200 µL sulfuric acid, to determine volatile fatty acids (VFAs) concentration. The remaining content was filtered (Ankom® filter F57 bags, Ankom Technology, Macedon, NY, USA) to calculate dry matter (DM), organic matter (OM), and NDF degradability.

Chemical analysis

Dry matter (DM; 930.04, AOAC, 2015), ash (942.05, AOAC, 2015), and neutral detergent fiber (NDF; Van Soest *et al.*, 1991) concentrations were determined in forages and residues from each fermentation bottle to calculate DM, OM, and NDF degradability (Blümmel and Lebzien, 2001). The ADF and NDF procedures are not ash-free. Total carbohydrate (TC) degradability was estimated by adding the NSC (assuming they are completely degraded) plus the NDF degradability. Crude protein (CP; 976.05, AOAC 2015), ether extract (EE; 930.09, AOAC 2015), acid detergent fiber (ADF; Van Soest *et al.*, 1991) and gross energy (6200 Calorimeter, Parr® 6510, Parr Instruments Company, Illinois, USA) were determined in the forages. Condensed tannins (CT) were quantified for the legumes by the butanol-HCL method (Terrill *et al.*, 1992). The CH_4 concentration at each incubation time and rumen VFAs were determined by gas chromatography (Shimadzu GC-2014, Shimadzu Corporation, Osaka, Japan) using a flame ionization detector (FID) according to Parra and Avila (2010), and Betancourt (2001), respectively.

Statistical analysis

Data were analyzed as a completely randomized blocks design in a 4×3 factorial arrangement, where species (clover, lotus, ryegrass, and kikuyu) and maturity stage (young, intermediate, and mature) were considered as main effects. The average of the three bottles was considered as analytical repetitions and the two paddocks (1 and 2) as block factor (replica). The GLM procedure of SAS® software, version 9.2 (SAS Institute, Inc, Cary, NC, USA) (2008) was used for variance analysis, and means were compared using the Tukey test with 5% significance. The relationship between total gas or CH_4 production and forage composition was assessed by multiple regressions, using the REG procedure of SAS® version 9.2 (SAS Institute, Inc, Cary, NC, USA).

Results

Nutritional composition of forages

As maturity increased, concentration of structural carbohydrates (NDF) increased ($p<0.001$), while EE and gross energy concentration decreased ($p<0.001$) for all forages (Table 1). The concentration of CT increased with legume maturity. Lotus contained 4.7 times more CT than clover, on average. The concentrations of CP, OM, and ash presented interaction between species and maturity stage (Table 1).

pH, degradability, VFAs, gas, and methane production

Interaction between species and maturity on pH was observed ($p<0.01$). For legumes, the pH was similar among maturity stages, while ferments involving young grasses had higher pH compared to intermediate or mature grasses. Legumes had slightly higher pH than grasses. Compared to legumes, total VFAs concentration (after 48 h incubation) was higher for ryegrass and intermediate for kikuyu ($p<0.05$). The molar proportions of VFAs and the acetate:propionate ratio were similar among maturity stages of legumes; but for grasses, especially kikuyu, the proportions of acetate and acetate:propionate ratio increased with maturity, whereas, propionate proportion decreased ($p<0.05$; Table 2). Degradability of DM, NDF, and OM decreased as the stage of maturity increased for all species, except for lotus, which presented higher degradability of DM, OM, NDF, and TC at intermediate age compared to young or mature stages (Table 2). Young forages produced less gas and methane per unit of degraded OM (dOM) than intermediate or mature forages ($p<0.05$; Figure 1), with the exception of lotus that had similar gas production among stages ($p>0.05$; Table 3). Lotus produced less methane per unit of dOM than ryegrass and clover ($p<0.05$), while kikuyu showed intermediate production (Figure 2).

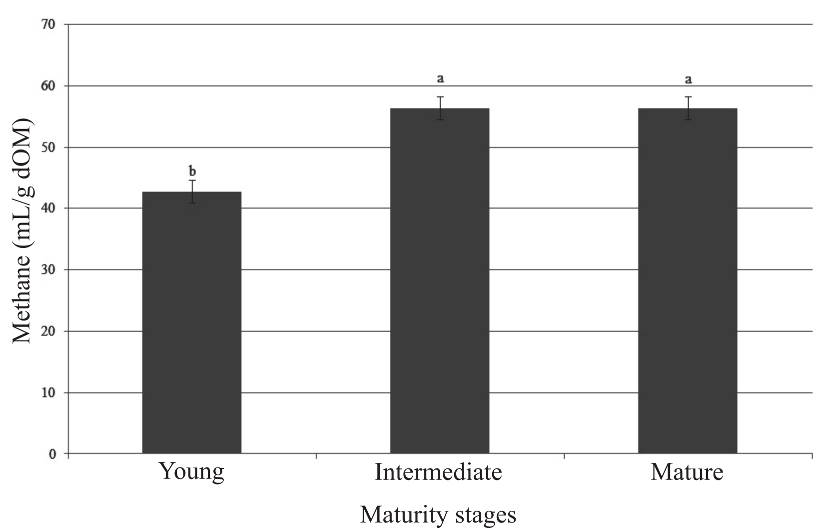

Figure 1. Effect of maturity stage on *in vitro* methane production of two grasses and two legumes (mL/g degraded OM).

Table 1. Chemical composition of two grasses and two legumes at different maturity stages (n = 24).

DM (%)	Clover			Lotus			Ryegrass			Kikuyu			p-value		
	Y	I	M	Y	I	M	Y	I	M	Y	I	M	Sp	St	Sp x St
CP	34.7	31.2	31.0	34.7^{a}	33.9^{a}	22.9^{b}	31.8^{a}	25.2^{a}	13.0^{b}	32.3^{a}	25.4^{a}	21.8^{b}	***	***	*
EE	5.9	5.5	5.0	2.9	2.2	1.4	6.0	4.1	3.7	5.2	5.4	4.2	***	*	ns
NDF	26.9^{b}	29.6^{ab}	39.6^{a}	31.3	31.9	34.6	41.3^{b}	46.5^{b}	59.5^{a}	41.1^{b}	50.0^{ab}	56.1^{a}	***	***	ns
ADF	14.6	15.9	22.6	17.1	20.6	23.3	22.4^{b}	25.5^{ab}	33.2^{a}	16.2^{b}	24.2^{ab}	25.0^{a}	***	***	ns
Hemicellulose	12.2	13.7	17.0	14.2	11.3	11.3	18.9	20.9	26.3	24.9	25.9	31.1	***	***	ns
Cellulose	12.3	13.4	17.2	10.8	13.2	15.3	20.6^{b}	22.7^{b}	29.3^{a}	14.6^{b}	21.7^{a}	23.1^{a}	***	***	ns
Lignin	2.3	2.5	5.3	6.3	7.5	8.0	1.8	2.8	4.0	1.6	2.5	1.9	***	***	ns
NSC	22.9^{a}	24.8^{a}	14.1^{b}	16.2^{b}	18.6^{b}	27.7^{a}	10.5	15.9	17.1	10.1	10.1	10.1	***	*	***
TC	0.1^{b}	0.3^{b}	2.5^{a}	4.2^{b}	4.2^{b}	5.2^{a}	ND	ND	ND	ND	ND	ND	***	***	***
Ash	9.6^{a}	8.5^{ab}	7.8^{b}	10.6^{a}	9.2^{ab}	8.2^{b}	10.5^{a}	8.3^{b}	6.7^{c}	11.0	10.1	9.9	***	***	**
CT	47.4	51.9	48.4	41.2^{b}	43.1^{b}	54.3^{a}	50.0^{b}	59.6^{b}	72.6^{a}	49.6^{b}	57.6^{ab}	64.3^{a}	***	***	***
OM	90.4^{b}	91.5^{ab}	92.2^{a}	89.4^{b}	90.8^{a}	91.8^{a}	89.6^{c}	91.7^{b}	93.3^{a}	89.0	89.9	90.1	***	***	**
Gross energy	4700.3	4709.1	4502.4	4559.3	4570.9	4280.4	4379.9	4176.6	4093.5	4239.9	4244.5	4128.7	***	***	ns

Y: Young; I: Intermediate; M: Mature; Sp: Species effect; St: Stage effect; SpxSt: Species and stage effects. Values followed by different superscript letters ($^{a, b, c}$) within rows indicate significant difference (* p<0.05; ** p<0.01). ns: Non-significant.

Table 2. Effect of maturity stage at 48 h of ruminal *in vitro* fermentation parameters of two grasses and two legumes.

Variable	Clover			Lotus			Ryegrass			Kikuyu			p-value		
	Y	I	M	Y	I	M	Y	I	M	Y	I	M	Sp	St	Sp x St
pH	6.8	6.8	6.8	6.8	6.8	6.8	6.7^{a}	6.7^{ab}	6.6^{b}	6.8^{a}	6.7^{b}	6.7^{ab}	**	**	**
Volatile fatty acids (VFAs)															
Total (mMol/L)	43.6	51.1	50.9	47.3	49.2	47.2	60.5	63.9	65.9	53.0	56.9	58.0	**	ns	ns
Acetate (Mol/100 Mol VFA)	62.3	64.2	66.2	63.4	61.2	63.7	62.6	62.0	65.0	60.8^{b}	67.6^{ab}	70.5^{a}	*	*	**
Propionate (Mol/100 Mol VFA)	22.6	20.5	19.7	22.6	22.4	21.0	22.7	21.9	19.2	23.8^{a}	20.4^{ab}	19.0^{b}	ns	*	**
Butyrate (Mol/100 Mol VFA)	7.7	7.8	8.2	8.0	9.1	9.1	8.2	9.2	9.6	6.7	6.9	8.2	**	ns	**
Acetate:propionate ratio	2.8	3.1	3.4	2.8	2.7	3.0	2.8	2.8	3.4	2.6^{b}	3.3^{ab}	3.8^{a}	ns	*	**
Digestibility (%)															
Dry matter (DM)	82.1^{a}	69.3^{b}	63.0^{c}	56.4^{b}	61.3^{a}	58.1^{ab}	72.5^{a}	71.8^{a}	63.5^{b}	71.1^{a}	68.1^{ab}	66.3^{b}	**	**	**
Neutral detergent fiber (NDF)	86.4^{a}	72.0^{b}	45.2^{c}	57.6^{a}	59.5^{a}	38.8^{b}	84.6^{a}	75.9^{a}	59.5^{b}	85.0^{a}	74.4^{b}	56.2^{c}	**	**	**
Total carbohydrates (TC)	87.5^{a}	79.5^{b}	49.8^{c}	60.7^{a}	60.3^{a}	49.4^{b}	83.6^{a}	77.6^{b}	63.3^{c}	84.4^{a}	75.9^{b}	57.2^{c}	**	**	**
Organic matter (OM)	86.5^{a}	72.7^{b}	65.8^{c}	58.3^{b}	63.5^{a}	60.5^{ab}	75.9^{a}	76.1^{a}	66.7^{b}	73.4^{a}	71.2^{ab}	69.2^{b}	**	**	**

Y: Young; I: Intermediate; M: Mature; Sp: Species effect; St: Stage effect; SpxSt: Species and stage effects. Values followed by different superscript letters ($^{a, b, c}$) within rows indicate significant difference (* p<0.05; ** p<0.01). ns: Non-significant.

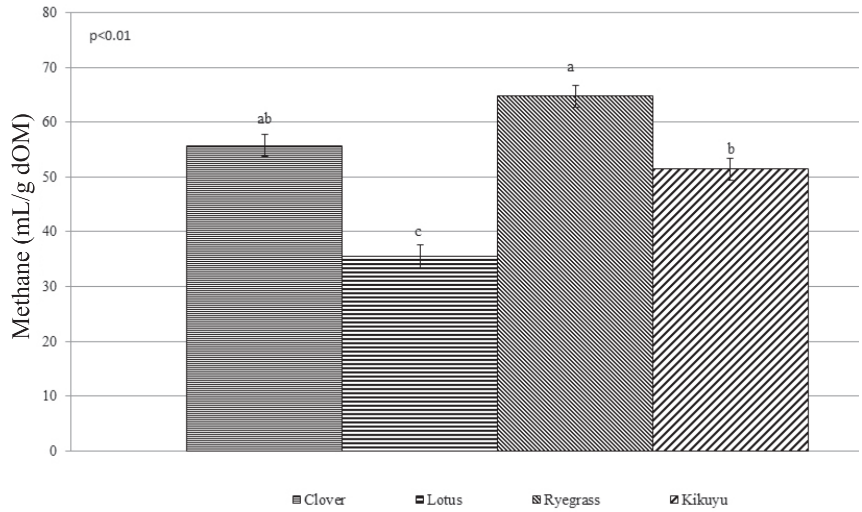

Figure 2. Ruminal *in vitro* methane production (mL/g degraded OM) after 48 h of fermentation of two grasses and two legumes.

Table 3. Effect of maturity stage on gas and methane production after 48 h of ruminal *in vitro* incubation of two grasses and two legumes.

Variable	Clover			Lotus			Ryegrass			Kikuyu			P-value		
	Y	I	M	Y	I	M	Y	I	M	Y	I	M	Sp	St	Sp x St
Gas production															
mL	31.6^{ab}	32.3^{a}	28.2^{b}	24.7	27.4	26.2	31.1	34.5	34.3	27.4^{b}	31.8^{a}	29.5^{ab}	*	*	*
mL/g dDM	434.9^{b}	524.8^{a}	504.5^{ab}	489.3	507.9	512.9	418.1^{b}	533.9^{ab}	601.7^{a}	424.5^{b}	521.4^{a}	495.9^{ab}	*	*	*
mL/g dOM	437.2^{b}	525.4^{a}	504.8^{ab}	502.4	515.3	515.9	488.3^{b}	527.6^{ab}	595.0^{a}	438.0^{b}	528.6^{a}	501.8^{ab}	*	*	*
Methane production															
mL	3.7	3.8	3.0	1.3	2.3	1.9	3.8	3.9	4.2	2.2	3.7	3.4	*	*	*
mL/g dDM	50.9	61.1	54.3	25.5	42.0	38.2	58.5	60.9	74.8	33.8	60.1	57.9	*	*	ns
mL/g dOM	51.2	61.5	54.4	25.2	42.9	38.4	59.7	60.5	73.9	35.1	60.2	59.0	*	*	ns
Methane:gas production ratio (%)	11.3	11.4	10.7	5.2	8.3	7.3	12.0	11.3	12.4	8.1	11.7	11.6	*	**	**

Y: Young; I: Intermediate; M: Mature; Sp: Species effect; St: Stage effect; SpxSt: Species and stage effects; dDM: Degraded dry matter. dOM: Degraded organic matter. Values followed by different superscript letters ($^{a, b, c}$) within rows indicate significant difference (* p<0.05; ** p<0.01; *** p<0.001; ns: Non-significant).

Regression analysis showed a positive linear relationship between CH_4 production (PCH_4) and cellulose (CEL) concentration (DM basis) and OM degradability (DOM; $R^2 = 0.67$, $p<0.01$):

$$PCH_4\ (mL) = -3.58 + 0.11 \times CEL\ (\%) + 0.07 \times DOM\ (\%) \quad \text{Eq. 1}$$

The percentages of CEL, ASH, and digestible TC (DTC) contents (DM basis) were linearly related to total gas production (TGP; $R^{2=}69$, $p<0.01$):

$$TGP\ (mL) = 17.44 + 0.37 \times CEL\ (\%) + 0.23 \times DCH\ (\%) - 1.27 \times ASH\ (\%) \quad \text{Eq. 2}$$

Discussion

Methane production and forage maturity

Similar to previous reports (Purcell *et al.*, 2011; Navarro-Villa *et al.*, 2011), we found that young forages incubated in a ruminal *in vitro* system

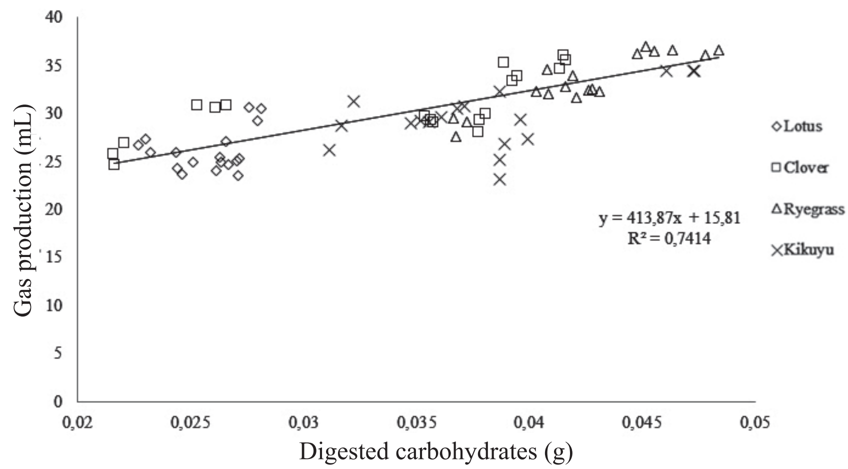

Figure 3. Relation of total *in vitro* gas production and degraded carbohydrates from two grasses and two forage legume species.

produced less CH_4 per unit of degraded organic matter compared to mature forages. This lower CH_4 production from young fodder has not been clearly explained. Moss *et al.* (2000) and Vargas *et al.* (2012) suggest that young forages have a higher concentration of NSC, which upon fermentation produce more propionate and consequently less CH_4. However, changes in NSC associated with maturity were not equal among species. While in kikuyu and clover, NSC decreased with maturity, this was not the case for ryegrass and lotus, in which NSC concentration increased with age. In our work, the proportion of propionate in fluid ferment was slightly higher for young grasses than for mature forages. This would imply that carbohydrate fermentation in young forages favors the pathway to propionate.

The literature suggests that fiber concentration is positively associated with CH_4 production (Hindrichsen *et al.*, 2005; Navarro-Villa *et al.*, 2011). Tiemann *et al.* (2008) found an increase in CH_4 production associated with hemicellulose fermentation in forages. In this experiment we found a positive correlation between cellulose concentration and CH_4 production, but not with hemicellulose.

In our study, young forages had higher protein content, but lower concentration of total carbohydrates than mature forages. Pelchen and Peters (1998) reported an inverse relationship between CP concentration and CH_4 production. In ruminal fermentation, dietary proteins are used for the synthesis of microbial protein or degraded to ammonium and VFAs (López, 2005). The first process does not produce CH_4, while in the second one the amount of CH_4 produced depends on the type and proportion of VFAs produced (Leng, 2011). In any case, the contribution of fermented protein to CH_4 production should be smaller. This fact has been recognized in feeding systems such as the Cornell Net Carbohydrate and Protein System (CNCPS), where it is assumed that the energy produced in VFA formation is generated mainly by carbohydrate fermentation (Sniffen *et al.*, 1992). Therefore, the production of CH_4 and VFAs in a ruminal *in vitro* system would be more closely associated with the fermentation of carbohydrates. In our study, the concentration of VFAs after 48 h of incubation was similar among maturity stages while degradability of DM and OM was higher for young forages. It is expected that VFAs concentration increases as degradability improves (Purcell *et al.*, 2011). However, in our experiment VFAs concentration was more closely associated with fermented carbohydrates than with total degradable OM ($r = 0.85$; $p<0.01$ vs 0.45, $p<0.01$, respectively). Finally, a lower production of CH_4 in young forages with higher protein concentration compared to mature forages may be also associated to higher concentration of nitrates in young forages. It has been shown that protein and nitrate concentration is greater in the early stages of maturity (Treviño and Hernández, 1978), which could reduce CH_4 production since nitrates capture part of the hydrogen produced during ruminal fermentation (Lee and Beauchemin, 2014).

The gas production technique has been used to estimate feed digestibility, where higher gas yields have been associated with higher digestibility (Lovett *et al*., 2004; Tavendale *et al*., 2005). In the present work, young forages, with the exception of lotus, presented lower gas production per unit of degraded OM than mature forages. Gas production was more closely related to fermented carbohydrates, which would largely explain the differences in CH_4 production.

Forage species

We found differences in CH_4 production associated with species, regardless of maturity stage. Lotus produced less CH_4 per unit of degraded OM and ryegrass produced more. Differences between species in CH_4 production in *in vitro* systems have been reported by other researchers (e.g., Singh *et al*., 2012), although the comparison between species is difficult due to variations in maturity stages. The lower methane production from lotus has been associated with the presence of tannins, both *in vitro* (Tavendale *et al*., 2005), and *in vivo* (Woodward *et al*., 2004). Tavendale *et al*., (2005) suggest that tannins may affect methanogenic populations. Other researchers suggest that condensed tannins can have bacteriostatic effects on some ruminal microorganisms, decreasing degradation of OM (Hess *et al*., 2008), protein (Waghorn, 2008) or fiber (Tiemann *et al*., 2008) and, therefore, decreasing CH_4 production. Minor degradation of these components would explain lower CH_4 and gas production, but could not explain lower gas production per unit of degraded OM, as found in our study. Regardless of maturity, we observed a lower ratio between CH_4 and gas production for lotus than for the other species, with the exception of young kikuyu. Other experiments, in which CH_4 concentration decreased in the gas, showed that, in many cases, there is an increase in H_2 concentration in the gas (Tavendale *et al.*, 2005). This suggests that part of the lower CH_4 concentrations is due to inhibition of CH_4 synthesis, and not to the use of H_2 for synthesis of other compounds (propionate, saturated fatty acids, reduce nitrate). This would explain why less CH_4 was produced, despite a lack of differences in the molar ratio of propionate.

In our study, CH_4 production per unit of degraded OM was comparatively higher for ryegrass than for legumes. In a meta-analysis, Archimède *et al.* (2011) suggested that legumes and grasses in temperate zones produce similar CH_4 *in vivo*. On the other hand, Navarro-Villa *et al.* (2011) reported lower CH_4 emission per unit of degraded organic matter in ryegrass with respect to clover, due to higher concentration of soluble carbohydrates, which increase the propionate:acetate ratio. Few studies have compared CH_4 production from kikuyu in relation to other forage species. In *in vivo* studies, Ulyatt *et al.* (2004) reported a greater CH_4 production in kikuyu vs other grasses in temperate zones. However, CH_4 production was not compared among maturity stages of different species in their study. In this sense, Archimède *et al.* (2011) reported that *in vivo* CH_4 production was 17% higher in C4 (kikuyu) in relation to C3 (ryegrass) forages, suggesting kikuyu would produce more CH_4 than ryegrass, which contrast with our results. However, care should be taken when comparing results from *in vitro* to *in vivo* assays. *In vitro* trials do not consider characteristics such as rate of passage, intake, and selectivity, which can affect CH_4 production (López, 2005).

Our work indicates that CH_4 percentage in the total amount of gas produced during fermentation is similar among species and stages of maturity, except for lotus and young kikuyu. These results suggest that gas production is not associated with the metabolic pathways of these species (C3 vs C4) or with the difference between grasses and legumes, but would be more closely associated with the concentration degradable carbohydrates. Considering that the pathways of carbohydrates degradation in the rumen share common intermediaries such as pyruvate, regardless of their type (sugar, hemicellulose, cellulose, starches, or pectins; Van Soest, 1994), the differences in the proportion of each VFA is associated with microorganisms using pyruvate (Stewart *et al.*, 1997). Murphy *et al.*, (1982) showed that a same carbohydrate can produce different proportions of VFAs, depending on ruminal pH and diet. Under the conditions of this study, where pH was more or less constant and substrates were fodder, it was expected that the type of microorganisms in the incubated fluid was similar among species. Gas production would, therefore, be closely associated with total carbohydrate degradation and not with differences in their proportion. Ryegrass, regardless of maturity, had a higher concentration of total degradable carbohydrates and a lower CP, which would explain its greater total gas production.

In conclusion, younger forages produce less CH_4 than mature ones, regardless of their species. Ryegrass produced more and lotus less methane per degraded OM. Methane production was explained mainly by variations in total gas production, since CH_4 proportion in gas among species and stages of maturity was similar, except for lotus and young kikuyu, for which it was lower. Condensed tannins in lotus, and probably nitrates accumulation in young kikuyu, could explain these differences. Total gas production was positively related to cellulose contents and total carbohydrate degradation.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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