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BRIEF COMMUNICATION: *In vitro* ruminal fermentation and methane production from maize silage mixed with high or low quality ryegrass

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Introduction

Methane is the main greenhouse-gas produced by New Zealand agriculture (Ministry for the Environment 2011). The methane loss from ruminants generally ranges from 4.5% to 9% of gross energy intake (Hammond et al. 2009). This indicates there is scope for lowering methane production. Replacing pasture forage with maize silage could decrease methane emission by altering fermentation patterns and by reducing rumen pH or ruminal retention time with the addition of starch to the diet (Beauchemin et al. 2008). However, Waugh et al. (2005) found that methane yield (g/kg DM intake) increased when pasture forage was substituted with up to 35% maize silage in dairy cows fed at similar intakes. Other literature suggests that methane yield in cattle responds in a quadratic manner when pasture forage is substituted with maize silage or grain (Blaxter & Wainman 1964; Arndt et al. 2010; Hassanat et al. 2012). This effect is associated with pasture forage quality (Kirkpatrick et al. 1997).

The objective of this study was to determine fermentation patterns and methane production of maize silage mixed at five levels with high or low quality ryegrass forage using the *in vitro* gas production technique.

Material and methods

Samples of freeze-dried maize silage with a composition of 30 g/kg ash, 29 g/kg fat, 69 g/kg crude protein (CP), 623 g/kg neutral detergent fibre (NDF) and 372 g/kg starch, ground through a 1 mm screen and mixed in ratios (w/w) of 0:100, 25:75, 50:50, 75:25 and 100:0 with either high or low quality ryegrass forage that had been oven-dried at 65˚C for 48 hours and ground through a 1 mm screen. The composition of the high quality ryegrass forage was 150 g/kg ash, 36 g/kg fat, 212 g/kg CP and 526 g/kg NDF, and the composition of the low quality ryegrass forage was 91 g/kg ash, 10 g/kg fat, 104 g/kg CP and 613 g/kg NDF. Each mixed sample (2 ryegrass qualities x 5 maize silage inclusion levels; n = 10) was subsequently subjected to an *in vitro* rumen incubation procedure. Increasing the proportion of maize silage in the mixture with either high or low quality ryegrass forage resulted in a decreased protein and increased starch content. Fibre content in the mixture was only increased when high quality ryegrass was substituted by maize silage.

Incubations were performed in three runs at AgResearch Grasslands (Palmerston North, New Zealand) using an automated *in vitro* gas production system with a gas chromatograph attached for automatic methane analysis in released gas as described by Muetzel et al. (2011). Rumen fluid was collected from a different fistulated cow in each run before the morning feeding. Donor cows were fed freshly cut pasture twice daily at 08:30 and 16:00 hours at 1.2x maintenance level. In each incubation run, duplicate samples (600 mg) from each treatment were incubated for 48 hours at 39°C in 120 mL bottles with 20 mL rumen liquid and 40 mL buffer (Muetzel et al. 2011). After 48 hours the contents were sub-sampled from each bottle for subsequent volatile fatty acid (VFA) analysis (Tavendale et al. 2005). The VFA data were used to calculate the hydrogen producing:reducing VFA ratios (H-VFA = (acetate + butyrate)/(propionate + valerate)).

Statistical analysis was performed by two-way analysis of variance with fixed effects of ryegrass quality, maize silage inclusion level and interaction between ryegrass quality and maize silage inclusion level using GenStat Version 13 (Payne et al. 2009). Incubation run (i.e. rumen liquid donor animal) was the experimental replicate (n=3). Polynomial contrast statement in GenStat was selected to test the linear effect of supplement inclusion level and Tukey statement was used for multiple-treatment comparison at $P < 0.05$. Significance was defined as $P < 0.05$.

Results and discussion

The 48 hour gas and methane production increased linearly ($P < 0.05$) with increasing maize silage inclusion in both ryegrass qualities. This effect was more pronounced in low quality ryegrass, which had lower ($P < 0.05$) gas and methane production compared with high quality ryegrass (Table 1). Starchy wheat-alfalfa pellets (60:40 w/w) had a higher gas and methane production than high and low quality ryegrass in the study of Muetzel et al. (2011), which is similar to the highest ranking of the starchy maize silage in this study. The increasing gas produciton with increasing starchy maize silage inclusion in this study is consistent with the general trend that *in vitro* gas production increases with increasing substrate starch content (Chai et al. 2004).

Volatile fatty acid production after 48 hours of incubation increased linearly ($P < 0.05$) with increased maize silage inclusion in low quality ryegrass, while

Table 1 Mean of three incubation runs for gas and methane production (mL/g incubated), volatile fatty acid (VFA) production (mmol/g incubated) and molar portions (mol/100 mol) after 48 hours of *in vitro* rumen incubation of high and low quality ryegrass (RQ) mixed with maize silage at five inclusion levels (IL). SED = Standard error of the difference; Total VFA production includes acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate; H-VFA = Ratio of hydrogen producing: reducing VFA ((acetate + butyrate)/(propionate + valerate)).

Type of measurement	Measurement	Ryegrass quality	Maize silage inclusion (%)					Ryegrass quality mean	Ryegrass quality		Inclusion level		RQ × IL	
			0	25	50	75	100		SED	P value	SED	P value	SED	P value
Gas emissions (mL/g)	Total gas	High	300	312	322	324		314	7	**<0.001**	11	**0.002**	16	0.14
		Low	248	267	286	310		278						
		IL mean	274^{a}	290^{ab}	304^{abc}	317^{bc}	323^{c}							
	CH_4	High	50.9	56.2	59.5	61.4		57.0	1.6	**0.04**	2.5	**<0.001**	3.5	0.79
		Low	46.9	50.9	54.2	59.2		52.8						
		IL mean	48.9^{a}	53.6^{ab}	56.8^{b}	60.3^{b}	60.8^{b}							
VFA production (mmol/g)	Total	High	7.22^{uv}	7.37^{v}	7.29^{uv}	7.17^{uv}		7.26	0.13	**<0.001**	0.20	0.07	0.29	**0.02**
		Low	5.79^{t}	6.31^{tu}	6.46^{tuv}	6.78^{tuv}		6.34						
		IL mean	6.50	6.84	6.87	6.98	7.13^{uv}							
Molar proportions (mol/100 mol)	Acetate	High	63.4^{xy}	62.8^{xy}	61.4^{uvw}	59.5^{u}		61.8	0.3	**0.001**	0.5	**<0.001**	0.7	**0.004**
		Low	67.1^{z}	64.3^{y}	61.9^{vw}	59.6^{uv}		63.2						
		IL mean	65.2^{e}	63.6^{d}	61.6^{c}	59.6^{b}	$56.9^{a,t}$							
	Propionate	High	19.0	17.5	16.5	15.7		17.2	0.6	0.56	1.0	**0.04**	1.4	0.98
		Low	18.0	16.9	16.1	15.7		16.7						
		IL mean	18.5^{b}	17.2^{ab}	16.3^{ab}	15.7^{ab}	15.3^{a}							
	Butyrate	High	10.8	13.1	15.5	18.4		14.4	0.6	0.85	1.0	**<0.001**	1.4	0.85
		Low	9.4	13.0	16.1	18.7		14.3						
		IL mean	10.1^{a}	13.0^{b}	15.8^{bc}	18.6^{c}	21.6^{d}							
	Valerate	High	2.46^{x}	2.21^{wx}	1.95^{vw}	1.71^{uv}		1.95	0.04	**<0.001**	0.06	**<0.001**	0.08	**<0.001**
		Low	1.25^{t}	1.36^{t}	1.39^{t}	1.43^{tu}		1.37						
		IL mean	1.85^{c}	1.78^{cd}	1.67^{bc}	1.57^{ab}	$1.44^{a,tu}$							
Ratios	H-VFA	High	3.47	3.85	4.19	4.51		4.00	0.18	0.18	0.29	**0.01**	0.40	0.32
		Low	3.98	4.23	4.48	4.60		4.32						
		IL mean	3.72^{a}	4.04^{ab}	4.34^{ab}	4.56^{ab}	4.78^{b}							
	CH_4/VFA	High	0.31	0.34	0.37	0.38		0.35	0.01	**0.04**	0.01	**0.002**	0.02	0.22
		Low	0.36	0.36	0.38	0.39		0.37						
		IL mean	0.34^{a}	0.35^{ab}	0.38^{bc}	0.38^{bc}	0.38^{bc}							

[abcde]Maize silage inclusion level means within a single row with differing superscripts are significantly different (P <0.05).
[tuvwxyz]Means within a block (interaction between grass quality and maize silage inclusion level; GQ × IL) of the same measurement with differing superscripts are significantly different (P <0.05).

VFA concentration remained similar when maize silage replaced high quality ryegrass (Table 1). Iso-butyrate, iso-valerate and caproate were similar among treatments with overall means of 1.45, 2.60 and 0.49 mol/100 mol VFA, respectively. Acetate and propionate proportion decreased (P <0.05) and butyrate proportion, H-VFA ratio and CH_4/VFA ratio increased (P <0.05) with increasing maize silage inclusion level with both ryegrass qualities (Table 1). The results suggest that the increased butyrate proportion seen with increasing maize silage inclusion may be driving higher methane production.

Results by Vibart et al. (2007) and Hildebrand et al. (2011) suggested that the effect of maize silage or grain inclusion level on rumen fermentation was influenced by outflow of unfermented material and/or fermentation end-products, which does not occur in the closed batch system in this study.

Increased rumen fermentation rate could reduce rumen pH resulting in reduced H-VFA ratios and methane production *in vitro* (Russell 1998). However, incubation medium pH will not reduce substantially, due to substrate used, in a buffered *in vitro* system used in this study. Also, the similar VFA and gas production for good quality ryegrass and maize silage suggest that they had similar substrate fermentability.

In summary, increasing maize silage inclusion in mixtures with both high and low quality ryegrasses increased methane production linearly *in vitro*, in a rumen system where there was no outflow and where rumen pH stayed constant. No quadratic responses to maize silage inclusion on methane production were observed in this study, contrary to previous cattle trials (Arndt et al. 2010; Hassanat et al. 2012). The results reported here suggest that fermentation rate and pattern are not directly responsible for the quadratic response *in vivo*. It is likely that factors such as the pH of the rumen environment or rumen outflow rate influence *in vivo* methane emission to a greater extent.

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