Effect on methane production of source of carbohydrate, and processing/variety of cassava leaf supplement, in an *in vitro* rumen incubation

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Abstract

The aim of this study was to compare effect of two source of protein varieties and process of cassava foliage and two sources of carbohydrate on methane production in an *in vitro* incubation medium inoculated with rumen fluid and using cassava foliage, cassava pulp and molasses for subtract. The design was a 2*2*2 factorial arrangement of the treatments with 4 replications. The quantity of substrate in each fermentation bottle was 12g DM to which was added 240 ml of rumen fluid (from slaughtered cattle) and 960 ml of buffer solution. The incubations were done in a simple *in vitro* incubation using one-liter bottles with gas collection by water displacement and methane measured by an infra-red methane gas detector. The incubation was for 24hours with measurements of total gas production and methane percentage in the gas at intervals of 6, 12, 18 and 24hours.

Methane production was lower when the substrate contained: cassava pulp rather than molasses as carbohydrate source; leaves from bitter rather than from sweet cassava variety; and cassava leaves that were in fresh form rather sun-dried. The methane percentage in the gas increased linearly with the length of the incubation.

Key words: *bitter, fresh, molasses, propionate, starch, sugar, sun-dried, sweet*

Introduction

Methane resulting from fermentative digestion of organic matter in the rumen represents a loss of dietary energy to the animal and is a significant contributor to world greenhouse gas emissions (Steinfeld et al 2006). These factors have led to a global search for strategies to mitigate methane emissions from ruminants.

In ruminants, hydrogen produced in the pathways of fermentation of organic matter to VFA with growth of cells is normally removed by the reduction of CO_2 to form methane. The net production of methane can be varied by: (i) introducing nitrate salts into the rumen as an alternative sink for the hydrogen, giving rise to ammonia instead of methane (Inthapanya et al 2011); (ii) increasing the escape of protein and fibrous carbohydrate from the rumen as when these are digested in the intestines (the protein) and/or fermented in the cecum-colon (the fibre) there is no production of

methane (Demeyer 1991); (iii) promoting the proportion of propionate in the rumen VFA as this captures more of the hydrogen with concomitant reduction in methane (Whitelaw et al 1984; Sangkhom et al 2017). Substrates rich in starch result in higher proportions of propionate in rumen VFA compared with those rich is sugars as in molasses (Preston 1993).

Cassava leaves are known to contain variable levels of condensed tannins which at moderate levels are known to have positive effects on the nutritive value of the feed by forming insoluble complexes with dietary protein, resulting in "escape" of the protein from the rumen fermentation (Barry 1999). Tannins are also reported to decrease methane production (Waghorn et al 2002), apparently through a direct toxic effect on methanogens. However, the other explanation is that increasing the rumen escape of protein leads to a shift of fermentation from the rumen to the cecum-colon with a concomitant reduction in methane (Phonethiep et al 2016).

In a recent study, Preston et al (2013) showed major effects of protein solubility on methane production in an *in vitro* incubation. Methane production was reduced by almost 50% when the protein source was fish meal (protein solubility 16.6%) compared with groundnut meal (protein solubility 76.2%) when sodium nitrate was the source of NPN and was 25% less when urea was the source of NPN. Cassava leaves from “bitter’ varieties contain protein of lower solubility, and give rise to more HCN in the rumen fermentation, than leaves from ‘sweet” varieties (Phuong et al 2012).

Cassava pulp is a by-product of cassava starch production and accounts for 10 to 15% of the original weight of fresh roots (Sriroth et al 2000). Recent research shows that its nutritive value is only slightly inferior to that of the original cassava root (Phanthavong et al 2014) and that it can be used as the basal diet for fattening cattle (Phanthavong et al 2016).

The purpose of the present study was: (i) to measure methane production in an *in vitro* rumen incubation with cassava pulp or molasses as the source of carbohydrate; and (ii) to confirm the reduction in methane production previously observed when the protein source was leaves from bitter rather than sweet cassava (Phuong et al 2012; Phanthavong et al 2015), in fresh form rather than as sun-dried meal (Phommasack et 2011).

Materials and methods

Location and duration

The experiment was carried out in the laboratory of the Research and Technology Transfer Center in Nong Lam University (NLU), Vietnam from May to July, 2015.

The *in vitro* system

The *in vitro* system (Photos 1 and 2) was based on the components and procedure reported by Inthapanya et al (2011).

Experimental design

Three factors were studied in a 2*2*2 factorial design with four replications. The factors were:

Carbohydrate source:

- Molasses
- Ensiled cassava pulp

Protein source: processing of cassava leaf

- Sun-dried leaves
- Fresh leaves

Protein source: variety of cassava

- Leaves from bitter cassava
- Leaves from sweet cassava

Procedure

Fresh leaves from sweet and bitter cassava varieties were harvested from plots in the experimental area of the Research Center in Nong Lam University. One portion of the leaves was dried in the shade for 24h then ground through a 1 mm sieve; the other portion was ground in the fresh state. Cassava pulp and molasses were sourced from cassava and sugar factories in Ho Chi Minh City. Urea (fertilizer grade) was obtained locally. All components were mixed together according to the proportions shown in Table 1.

Table 1. The proportions of the ingredients in the substrate (DM, g)

	Molasses				**Cassava pulp**			
	Sweet cassava		**Bitter cassava**		**Sweet cassava**		**Bitter cassava**	
	Dried	**Fresh**	**Dried**	**Fresh**	**Dried**	**Fresh**	**Dried**	**Fresh**
Cassava leaf	2.76	2.76	2.76	2.86	3.60	3.60	3.60	3.60
Molasses	8.97	8.97	8.97	8.97				
Cassava pulp					8.15	8.15	8.15	8.15
Urea	0.27	0.27	0.27	0.27	0.25	0.25	0.25	0.25

Total DM	12	12	12	12	12	12	12	12

The substrates (12g DM) were put in the incubation bottle to which were added 960ml of buffer solution (Table 3) and 40ml of rumen fluid. The rumen fluid had been taken at 10-11pm in the city abattoir from a buffalo immediately after the animal was killed. A representative sample of the rumen contents (including feed residues) was put in a vacuum flask and stored until 8-9am the following morning when the contents were filtered through one layer of cloth before being added to the incubation bottle. The gas space inside the bottle was then flushed with carbon dioxide prior to the incubation at 38Â°C for 24h.

Table 2. Ingredients of the buffer solution (Tilly and Terry 1963).

	CaCl2	**NaHPO4.12H2O**	**NaCl**	**KCl**	**MgSO4.7H2O**	**NaHCO3**	**Cyst**
(g/liter)	0.04	9.3	0.47	0.57	0.12	9.8	0.2

Photo 1. The *in vitro* system

Photo 2. Using “plasticine” to seal the ju
inlet tube
and the bottle used to measure ga
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Measurements

The gas volume was measured at 6, 12, 18 and 24 by water displacement from the receiving bottle suspended in water which was calibrated at intervals of 50ml. On each occasion, after measuring the volume, the gas was ejected from the receiving bottle though a tube attached to a Crowcom meter (Crowcom Instruments Ltd, UK) fitted with an infra-red sensor to measure methane (Photo 3). Undigested substrate at the end of the incubation was filtered by cloth and was then dried at 100$^{Â°}$C for 24h to determine residual DM.

Photo 3. Measurement of methane with the Crowcom meter

Photo 4. The substrate residue filtered through cloth

Data analysis

Data were analyzed by the General Linear Model in the ANOVA program of the Minitab software (Minitab 2010). Sources of variation in the model were: replication, carbohydrate source, processing, variety, interaction processing*variety and error.

Results

There were linear trends in gas production with increasing 6h periods of incubation: negative for gas production (Table 3; Figure 1) and positive for content of methane in the gas (Figure 2). Gas production was higher on molasses than on cassava pulp, higher for fresh than sun-dried cassava leaves (Figure 3), and higher for sweet cassava than for bitter cassava variety (Figure 4). The percentage of methane in the gas was lower for fresh than for dry leaves (Figure 5) and for the bitter variety compared with the sweet variety (Figure 6). The DM mineralized was higher for molasses than for cassava pulp, and higher for fresh than for sun-dried cassava leaves, with no apparent difference between cassava varieties. Methane production per unit substrate DM mineralized was lower for cassava pulp than for molasses, lower for fresh than for sun-dried cassava leaves and lower for the bitter than the sweet cassava variety (Figure 7).

Table 3. Effect of source of carbohydrate, processing and variety of cassava leaf, on mean values for gas methane production and DM digested

	Carbohydrate		p	Process		p	Variety		p
	Molasses	Cassava pulp		Sun-dry	Fresh		Bitter	Sweet	
Gas, ml									
0-6h	933	681	<0.001	830	785	0.198	790	832	0.331
6-12h	800	663	<0.001	744	719	0.272	707	756	0.032
12-18h	696	609	<0.001	672	634	0.037	631	675	0.017
18-24h	384	536	<0.001	493	427	<0.001	451	469	0.025
Total	2813	2967	<0.001	2739	2566	0.005	2380	2724	0.016

DMM, %#	71.7	55.1	<0.001	61.4	65.4	0.002	63.3	63.5	<0.001
Methane, %									
0-6h	12.2	11.9	0.21	12.5	11.6	<0.001	11.6	12.5	<0.001
6-12h	16.8	15.7	<0.05	17.2	15.3	<0.001	15.2	17.3	<0.001
12-18h	19.9	18	<0.001	19.8	18.1	<0.001	18.1	19.8	<0.001
18-24h	23.5	22.5	<0.001	23.8	21.8	<0.001	22	23.6	<0.001
Methane									
Total, ml	477	380	<0.001	482	409	<0.001	413	479	<0.001
% of total gas	17.0	14.0	<0.05	17.6	15.9	<0.001	16.0	17.5	<0.001
ml/g DMM	61.2	57.7	<0.001	65.4	52.1	<0.001	54.3	62.9	<0.001

#Dry matter mineralized

Figure 1. Effect of carbohydrate source on gas production in an *in vitro* rumen incubation

Figure 3. Effect of processing (fresh or dried) cassava leaves on gas production in an *in vitro* rumen incubation using leaves from a bitter or sweet variety an *in vitro* rumen incubation with molasses or cassava pulp as carbohydrate source

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Figure 5. Effect of processing leaves from sweet and bitter cassava on percent methane in the gas in an *in vitro* rumen incubation with molasses or cassava pulp as carbohydrate source

Figure 7. Effect on methane production of sou
and processing and variety of cassava le

Discussion

The reduction in methane production when the cassava leaves were from the bitter as opposed to the sweet variety of cassava agrees with the findings of Phuong et al (2012) and Phanthavong et al (2015). These authors cited the research of Smith et al (1985) and Cuzin and Labat (1992) which showed a toxic effect of cyanogens on methanogenic bacteria. A similar explanation probably applies to the reduction in methane production when fresh as opposed to dried cassava leaves were the source of protein in the substrate. A reduction in in methane production with fresh as opposed to dried cassava leaves was also reported by Phommasack et al (2011). According to Phuc et al (1996), drying of cassava leaves reduced the potential HCN levels by 89% compared with fresh leaves. Similar effects on potential HCN levels from drying cassava leaves were reported by Khieu Borin et al (2005) and Chhay Ty et a (2007).

It is well established that replacing a starch-based feed (maize) with one rich in soluble sugars (molasses) reduces the molar proportions of propionic acid in the rumen fermentation (Preston no date); and that feeds that support increasing proportions of propionic acid in the rumen fermentation result in decreased production of methane (Sangkhom et al 2017; Syahniara et al 2016). We have no data on the molar proportions of the rumen VFA in the present experiment but it is to be expected that propionate levels would have been higher on the cassava pulp treatment and that this would account for the lower methane production when cassava pulp replaced molasses as the carbohydrate source.

Conclusions

- Methane production in an in vitro incubation was lower when the substrate contained:
    - Cassava pulp rather than molasses as carbohydrate source
    - Leaves from Bitter rather than from Sweet cassava variety as protein source
    - Fresh rather sun-dried cassava leaves
- Methane percentage in the gas increased linearly with the length of the incubation

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Go to top