EFFECT OF SUPPLEMENTS OF YEAST (SACCHAROMYCES CEREVISIAE), *RICE DISTILLERS' BY-PRODUCT AND FERMENTED CASSAVA ROOT ON METHANE PRODUCTION IN AN IN VITRO RUMEN INCUBATION OF ENSILED CASSAVA ROOT, UREA AND CASSAVA LEAF MEAL*

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3.1.1. ABSTRACT

An *in vitro* rumen incubation was carried out to determine effects on methane production of supplementing ensiled cassava root, urea and cassava leaf meal with rice distillers' byproduct, fermented cassava root and yeast (*Saccharomyces cerevisiae*). The four treatments in a completely randomized design were: CTL: No supplement; RDB: 4% (in DM) rice distillers' byproduct; FCR: 4% (in DM) urea-fermented cassava root; Yeast: 1% (in DM) commercial yeast. The quantity of substrate in each fermentation bottle was 12g DM to which was added 240ml of rumen fluid (from slaughtered cattle) and 960ml of buffer solution. The incubations were done using one-liter water bottles with gas collection by water displacement. Measurements of total gas production and methane percentage in the gas were made at intervals of 0-3, 3-6, 6-12, 12-18 and 18-24h. The hourly rate of gas production increased to a maximum in the 6-12h of incubation interval and then decreased linearly. In contrast, the proportion of methane in the gas increased linearly with incubation interval from the beginning until the final 18-24h interval. Total gas production was highest for the fermented cassava root additive, followed by the rice distillers' byproduct with lowest values for the control and yeast treatments. The methane content of the gas was highest for the control treatment, followed by the fermented cassava root and yeast with the lowest value for rice distillers' byproduct, for which the overall reduction in methane was of the order of 25%. Methane production per unit DM digested showed a similar trend as the methane percentage in the gas. It is suggested that the benefits from brewers' grains and rice distillers' byproduct, in reducing methane production in the rumen fermentation, both *in vitro* and *in vivo*, are the indirect effects of these additives increasing the proportions of propionic acid in the rumen VFA.

Key words: *β-glucan, greenhouse gas, hydrolysis, propionate, urea*

3.1.2. 1. INTRODUCTION

Agriculture is an important source of greenhouse gas mainly through emissions of methane from enteric fermentation in ruminants and decomposition of manure (Gerber *et al*., 2013). Ruminants are estimated to produce up to 95 million tons of CH_4 annually, mainly from enteric fermentation and to a lesser extend from decomposition of manure (O'Mara, 2011; Patra, 2014). Strategies to reduce these emissions should first address the need to increase productivity of ruminant livestock; secondly, the feeding systems that lead to increased ruminant productivity are those that lead to increased proportions of propionic acid; and thirdly, the escape of protein from the rumen contributes amino acids directly to the animal through enzymatic digestion of protein in the intestines. The additional advantage of this process is that fibrous feed particles that escape the rumen attached to the protein will still be fermented to useful end products but in the cecum-colon section of the ruminant digestive tract in which the fermentation process does not produce methane (Demeyer, 1991).

In countries located in temperate latitudes, cereal crops such as maize and barley are the choice of feeds for intensifying ruminant production. Maize is grown in tropical latitudes but yields do not compete with those in temperate climates. By contrast, cassava **(***Manihotesculenta Crantz*) is a crop that originated in the tropics (in the Caribbean) and is

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now grown in over 90 countries world-wide (Lebot, 2009). Of importance in a warming world is that cassava is potentially highly resilient to future climatic changes and according to Jarvis *et al.* (2012) "could provide Africa with options for adaptation whilst other major food staples face challenges". Regarding the use of cassava for cattle production, these developments have been the driving force for a series of researches directed at optimizing the use of both the pulp and the fresh cassava root as the basis of intensive systems of livestock production, especially the fattening of local cattle (Phanthavong *et al*., 2014; 2015; 2016; 2017; Inthapanya *et al*., 2016). An additional advantage of cassava over cereal crops such as maize is that the foliage has proved to be a valuable source of bypass protein such that the cassava plant becomes a source both of highly digestive carbohydrate (from the root) as well as protein (from the foliage). The only additional features needed are a source of fermentable nitrogen (available locally as fertilizer grade urea) and minerals.

The development of cattle feeding systems based on cassava has stimulated an important outcome, namely how to manage the potential toxicity linked with the presence throughout the plant of cyanogenic glucosides that give rise to hydrocyanic acid when exposed to favorable conditions (eg: appropriate enzymes) in the plant itself or within the digestive of animals that consume it. Recent research, much of it in the laboratory of the senior author of this paper, has shown that the potentially toxic cyanogenic glucosides in cassava can be neutralized by supplementing the animal diet with small quantities of locally available by-products from fermentation industries, specifically from the production of beer which gives rice to "brewer' grains"; and the artisan distillation of fermented rice to make an alcoholic wine, which produces a byproduct known in Lao PDR as "Quilao", in Vietnam as "Hem" and in Cambodia as "Bar Ran". Brewers' grains have been shown to aid directly in the detoxification of HCN in cases where forage of a "bitter" (high HCN potential variety) was fed (Binh *et al*., 2017). Both brewers' grains and rice distillers' byproduct, fed at less than 5% of the diet, have resulted in reduced production of rumen methane and improved growth rates in cattle fed ensiled cassava pulp-urea of ensiled cassava root-urea as basal diet (Keopaseuth *et al*., 2016; Sengsouly and Preston, 2016; Inthapanya *et al*., 2017).

Access to brewers' grains is limited to farmers living in close proximity to the beer factory. Rice distillers' byproduct is more widely available in rural areas, but supplies are limited. For these reasons, research to identify alternatives to both brewers' grains and rice distillers' byproduct are considered to be of high priority. As both brewers' gains and rice distillers' byproduct are products of fermentation by yeast (specifically *Saccharomyces cerevisiae*), it was decided to evaluate: (i) a commercial source of yeast commonly available in local markets; and (ii) cassava root enriched by yeast fermentation with additional sources of nitrogen (urea) and phosphorus (di-ammonium phosphate – DAP). The purpose of the present study was to determine effects on CH_4 production in an *in vitro* rumen fermentation when yeast, rice distillers' byproduct, or fermented cassava root, were added in small amounts (4% DM basis) to a basal substrate of ensiled cassava root supplemented with urea and cassava leaf meal.

2. MATERIALS AND METHODS

2.1. Location and duration

The experiment was conducted in the laboratory of the Department of Animal Science, Faculty of Agriculture and Forest Resources, Souphanouvong Uni., LuangPrabang, Lao PDR.

2.2. Treatments and experimental design

The experimental design was completely randomized (CRD), 4 treatments and each with 5 replicates. The treatments were:

CTL: Ensiled cassava root

RBD: CTL + rice distillers' byproduct at 4% of DM

FCR: CTL+ fermented cassava root at 4% of DM

Yeast: CTL+ yeast at 1% of DM

All the treatments included urea (2% of root DM), cassava leaf meal from a bitter variety (25% of substrate DM) and S-rich minerals (1% of DM substrate).

2.3. *In vitro* rumen fermentation system

The **in vitro** rumen fermentation system (Diagram 1) was the same as that used by Sangkhom *et al.* (2011). The water bottles (capacity 1.5liters) were used for the fermentation and collection of the gas. These were connected by plastic tube (id 4mm) to a similar bottle which received the gas (the bottom of which had been removed) and which was suspended in a larger bottle (3liters capacity) partially filled with water, so as to collect the gas by water displacement. The bottle that was suspended in water was calibrated at 50ml intervals to indicate the volume of gas.

2.4. Experimental procedure

The cassava roots and leaves were collected from the Souphanouvong University farm; the roots were chopped into pieces around 1-2cm of length, ground in a liquidizer, and then stored in a plastic bag for ensiling over 7 days. Cassava leaves were chopped into small pieces around 1-2cm in length, then dried in the oven at 80ºC for 24h before grinding. The rice distillers' byproduct was collected from a farmer accustomed to produce "rice wine alcohol".

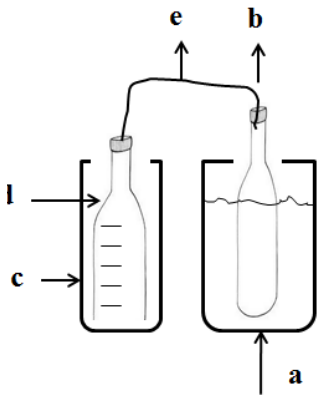

Fig 1. A schematic view of apparatus to measure in an in vitro rumen fermentation

a) Water bath
b) Fermentation bottle (1.5liters)
c) Water storage reservoir (3 liters)
d) Gas collection bottle (1.5liters)
e) Plastic tube (id: 4mm)

Buffer solution was prepared before incubation by mixing 0.04g $CaCl_2$, 9.30g; $NaHPO_4.12H_2O$, 0.47g; NaCl, 0.57g; KCl, 0.12g; $MgSO_4.7H_2O$, 9.80g; $NaHCO_3$, 0.25g of Cysteine and distil-water up to one litter together (Tilly and Terry, 1963).

The procedure for producing "fermented cassava root" was as follows: the roots were chopped and steamed for 30 minutes, allowed to cool for 15mintutes and then mixed with di-ammonium phosphate (DAP) at 0.8, 3% of yeast (*Saccharomyces cerevisiae*) and 2% of urea (all on DM basis) prior to being fermented in a closed plastic bag for 7 days. Representative samples (12g DM) of the substrates was put in the incubation bottle to which was added 0.96l of buffer solution and 240ml of rumen fluid (obtained from a slaughtered cow) prior to filling each bottle with carbon dioxide. The bottles were incubated at 38ºC in a water bath for 24h.

2.5. Data collection and measurements

The gas volume was recorded over intervals of 0-3, 3-6, 6-12, 12-18 and 18-24h. The methane concentration in the gas collected over each interval was measured with a Crowcon infra-red analyzer (Crowcon Instruments Ltd, UK). At the end of the incubation, the remaining substrate was filtered through cloth and the solid residue dried at 100°C to determine the DM digested.

2.6. Chemical analyses

Samples were analyzed for DM, ash, CP and CF according to AOAC (1990) methods. The solubility of the protein in diet ingredients was determined by extraction with M NaCl (Whitelaw and Preston, 1963).

2.7. Statistical analysis

The data were analyzed by the general linear model option of the ANOVA program in the Minitab software (version 16.0). In the model the sources of variation were: treatments, and error. Tukey's pair-wise comparisons was used to determine the differences between treatments when the P value of F test <0.05. The statistical model used was: $Y_{ij} = \mu + T_j + e_{ij}$. *Where: Y_{ij} was the dependent variable, μ was the overall mean, T_j was the effect of treatment, and e_{ij} was random error.*

3.1.3. 3. RESULTS AND DISCUSSION

3.1. Chemical composition

The values for DM, CP and solubility of the protein in the rice distillers' by-product (Table 1) were similar to previous studies (Luu Huu Manh *et al.*, 2009; Sangkhom *et al*., 2017) and the cassava root after fermentation with yeast, urea and DAP had a similar level of CP as reported for the same procedure by Vanhnasin *et al*. (2016) and Manivanh *et al.* (2016).

Table 1. Chemical composition of substrate components

Items	DM, %	CP	CF	Ash	Soluble CP
		As % of DM			% of total CP
Ensiled cassava root	32.1	2.51	1.12	0.88	9.22
Fermented cassava root	39.9	10.4	1.13	0.87	11.6
Bitter cassava leaf meal	90.3	19.0	15.2	6.14	31.3
Rice distillers' by-product	7.46	24.2	2.29	4.45	37.9
Yeast		45.0			32.4

3.2. Gas production

Gas production was higher for fermented cassava root than rice distillers' by-product and yeast or control treatment (Table 2), whereas the CH_4 content of the gas was differed among treatments with highest values for the control treatment, followed by the fermented cassava root, yeast and rice distillers' by-product, respectively.

Table 2. Means for gas production, methane, digestibility and methane per unit substrate digested

Items	CTL	FCR	Yeast	RDB	SEM	P
Gas production, ml: 0-3hr	360^{a}	420^{b}	370^{a}	380^{a}	11.7	0.012
3-6hr	510^{a}	700^{b}	520^{a}	580^{a}	36.7	0.008
6-12hr	760^{a}	1070^{b}	820^{a}	950^{c}	36.7	<0.001
12-18hr	640^{a}	940^{b}	690^{ac}	720^{c}	20.6	<0.001
18-24hr	470^{a}	630^{b}	480^{a}	600^{b}	19.0	<0.001
Methane,%: 0-3hr	8.4^{a}	7.8^{a}	6.4^{b}	6.4^{b}	0.28	0.001
3-6hr	11.4^{a}	10^{b}	8.4^{c}	7.6^{c}	0.35	<0.001
6-12hr	18.6^{a}	17.6^{a}	15^{c}	12.6^{b}	0.70	<0.001
12-18hr	22^{a}	21.2^{a}	19.6^{c}	17^{b}	0.39	<0.001
18-24hr	24.2^{a}	23.6^{a}	21.2^{c}	19^{b}	0.37	<0.001
Total gas, ml	2740^{a}	3760^{b}	2880^{a}	3230^{c}	62.7	<0.001
Total methane, ml	484^{b}	637^{c}	427^{a}	426^{a}	11.5	<0.001
Methane, % total gas	17.7^{d}	16.9^{c}	14.8^{b}	13.2^{a}	0.227	<0.001
DM digestibility, %	59.2^{a}	69.4^{b}	61.1^{a}	65.2	0.83	<0.001
CH4, ml/ g DM digested	68.4^{c}	76.5^{d}	58.2^{b}	54.4^{b}	1.76	<0.001

Where: Values on the same row with different superscripts differ (P<0.05)

3.3. Effect of incubation interval

The rate of gas production increased to a maximum in the interval 6-12h of incubation then decreased linearly. In contrast, the proportion of methane in the gas increased linearly with incubation interval (Table 2).

Table 3. Mean values for percent methane in the gas, and gas production per hour, during successive intervals of the fermentation

Items	0-3	3-6	6-12	12-18	18-24	SEM	P
Methane, %	7.25	9.35	16.0	20.0	22	0.203	<0.0001
Gas, ml/h	128	193	150	125	91	3.8	<0.0001

3.4. Effect of additives

Total gas production was highest for fermented cassava root, followed by RDB with lowest values for the yeast and control treatments (Table 3). The CH_4 content of the gas differed among treatments with highest values for the control treatment, followed by the fermented cassava root, yeast and RDB. On the RDB treatment the overall reduction in percent methane was of the order of 25%. CH_4 production per unit DM digestibility showed the same trend as the CH_4 percent in the gas (Table 3), respectively.

The increases in CH_4 production in the gas with duration of fermentation time, indicative of the transition to a secondary fermentation of the VFA to methane, supports earlier findings using a similar *in vitro* incubation system but with different substrates (Le Thuy Binh Phuong *et al.,* 2011; Outhen *et al.,* 2011; Sangkhom *et al*., 2011; Thanh *et al*., 2011).

The beneficial effect of the small quantity (4% of substrate DM) of rice distillers' by-product in reducing CH_4 production from rumen fermentation is similar to the response reported by Sangkhom and Preston (2016) when brewers' grains and rice distillers' by-product were added to *in vitro* incubations of ensiled and fermented cassava roots. Addition of yeast at 1% of the substrate also reduced CH_4 production but was slightly less effective than the rice distillers' by-product. By contrast, an attempt to simulate some of the features of RDB by fermenting cassava root with yeast, urea and di-ammonium phosphate had no effect on CH_4 production in the *in vitro* fermentation. It has been postulated that the benefits of yeast-based additives in improving human health and growth rates of animals are related to the β-glucan present in the yeast cell wall and their effect in stimulating the immune system (Dritz *et al.,* 1995; Hanh *et al*., 2006; Novak and Vetvicka, 2002; Waszkiewicz-Robak, 2013).

In ruminant systems it is probable that their effects are modulated through effects on microbial ecosystems in the rumen and/or lower down the digestive tract. Thus alleviation of hydrocyanic acid toxicity, in cattle fed cassava foliage from a variety rich in HCN precursors, has been suggested as being due to the β-glucans in brewers' grains supporting biofilm-based fermentations in the rumen that favored detoxification of the HCN (Inthapanya *et al.,* 2017). A shift in the microbial fermentation towards propionate at the expense of acetate production increases the overall yield of metabolisable energy. It also increases availability of glucogenic substrate which is often critically low in some feeds (Preston and Leng, 1987), H_2 produced when acetate is the end product of organic matter fermentation is converted to CH_4 and thus reduces overall metabolisable energy. Improvements in growth rate and feed conversion in cattle supplemented with brewers' grains or rice distillers' product are thus to be expected from the combined benefits of increased availability of both glucose precursors from propionic acid and increased total metabolisable energy as a result of decreased CH_4 production in the rumen (Sangkhom and Preston, 2016; Sengsouly and Preston, 2016; Binh *et al.,* 2017). Brewers' grains and rice distillers' by-product is the presence of yeast which has been subjected to heating (100˚C) under acid conditions during the process of distilling off the ethanol as is standard practice in production of beer from barley (brewers' grains) and from polished/broken rice (rice "wine"). It is then assumed that it is the β-glucan derived from the cell walls of the yeast (and of the barley), which modifies microbial activities in the rumen biofilms (Leng, 2014) favouring higher proportions of propionic acid in the rumen VFA. β-glucans are present in the cell wall of barley (Havrlentová and Kraic, 2006) and yeast (Waszkiewicz-Robak, 2013). An important issue is the need, or otherwise, to isolate the β-glucan which, as described by Nguyen Thi Thuy and Nguyen Cong Ha (2016), required high

pressure homogenization to break the yeast cell wall followed by acid, then alkaline hydrolysis. The results from our experiment, in which there were no effects on CH_4 by supplementing the substrate with fermented cassava root, partially support the hypothesis that breakage of the yeast/barley cell walls, followed by acid hydrolysis, are necessary first steps in facilitating the action of the β-glucan. The positive effect of the commercial yeast "starter" in decreasing CH_4 production implies that some of the β-glucan in this product may have been in the free-state. However, the greater impact of the rice distillers' by-product in reducing CH_4 production would probably have been facilitated by the degree of hydrolysis of the yeast cells that were likely to have occurred in the process of distilling the ethanol. The positive effect of rice distillers' by-product in reducing CH_4 production *in vitro* mirrors the results obtained *in vivo* when local cattle were fed basal diets of ensiled or fermented cassava root supplemented with urea and fresh cassava foliage (Sengsouly *et al.*, 2016; Sangkhom *et al.*, 2016). In both these experiments the reduction in CH_4 emissions were directly linked with improved growth rates and better feed conversion.

3.1.4. 4. CONCLUSIONS

Obtained results indicated that (i) the rate of gas production increased to a maximum in the 6-12h incubation interval and then decreased linearly. In contrast, the proportion of methane in the gas increased linearly with incubation interval from the beginning until the final 18-24h interval; (ii) Total gas production was highest for the fermented cassava root additive, followed by the rice distillers' by-product with lowest values for the control and yeast treatments; (iii) The CH_4 content of the gas was highest for the control treatment, followed by the fermented cassava root and yeast with the lowest value for rice distillers' by-product, for which the overall reduction in CH_4 was of the order of 25%; and (iv) CH_4 production per unit DM digested showed the same trend as the CH_4 percentage in the gas.

It is suggested that the reported benefits from brewers' grains and rice distillers' by-product in reducing CH_4 production in the rumen fermentations, both *in vitro* and *in vivo*, are the indirect effects of these additives increasing the proportions of propionic acid in the rumen VFA.

3.1.5. ACKNOWLEDGMENTS

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