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Evaluation of Pre-ingestive Citronella Residues using Ruminal *In Vitro* Techniques

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Abstract

Citronella residues (CR) have the potential to be used as an alternative to the fiber diet of ruminants. This study reports on the effects of pre-ingestive CR using ammonia (4 % dry matter (DM)) and fermentation (6 % DM) on *in vitro* rumen fermentation, metabolism, and methane (CH_4) production. Four CR levels of 0, 25, 75, and 100 % DM were used. Each level was repeated 3 times and a complete randomized design method was used. The results showed that pre-ingestive CR significantly increased the pH rumen fluid but decreased ammonia-nitrogen concentration, total iso-volatile fatty acid (iso-VFA) production, and protozoa population ($p < 0.01$). The pre-ingestive CR significantly decreased the acetic acid composition and rumen microbial protein synthesis ($p < 0.05$), and significantly increased the proportion of propionic acid, n-butyric acid, and iso-valeric acid ($p < 0.05$). The total VFA production and rumen CH_4 production did not significantly change ($p > 0.05$). In conclusion, pre-ingestive CR was compatible as a basal diet for ruminants.

Keywords: Ammonia, Citronella residues, Evaluation, Fermentation, *In vitro* rumen, Methane

Introduction

The utilization of feeds using local ingredients has become increasingly important in the livestock sector in recent years. It is a strategy to increase feed resilience by using abundant local feed resources to maintain animal production. Citronella grass (*Cymbopogon nardus*) is widely distributed in mainland Indo-China and the leaves are used as a raw material for the production of essential oils [1,2]. In south-east Asian countries such as Indonesia, citronella has developed into a leading plant product over the last decade, with an average plantation area of 19,370 ha and a biomass production of 2,340 tons/year [3]. The total agricultural yield of citronella is approximately 3.51 kg/plant/year [4]. Furthermore, Sari *et al.* [5] showed an analysis of the crude protein and neutral detergent fiber contents in the citronella residue (CR), which were approximately 5.82 and 73.67 % of the dry matter (DM), respectively. The phytochemical profiles of *C. nardus* leaves showed the presence of condensed tannins and several phenol components, namely caffeic acid, *p*-coumaric acid, and ferulic acid, which had biological activity as antimicrobial compounds [6]. The residue, a byproduct of the distillation process and citronella extraction, can be used as an alternative source of fiber in the daily basal diet of ruminants. Due to its potential availability and nutritional content, studies evaluating CR as a component of ruminant diets are needed.

It is of interest to improve the nutritional value of CR to enhance the characteristics of rumen fermentation, which directly affects animal production. The attempt to develop strategies to improve nutritional quality of CR and reduce rumen methane (CH_4) emissions from agricultural products has led to the application of pre-ingestive technology. Feed processing techniques using pre-ingestive methods with ammonia and fermentation have been shown to increase the utility of agricultural waste-based feed [7,8]. Citronella supplementation in ruminants has been reported to have beneficial effects. Wanapat *et al.*

[9] reported that citronella meal supplementation was able to increase nitrogen utilization and eliminate rumen protozoa populations. Sari *et al.* [5] conducted a comparative study between CR and freshly cut citronella, and showed that the *in sacco* digestibility, the DM level, and the organic matter level in the residue were higher. Vázquez-Carrillo *et al.* [10] observed an antimethanogenic effect of citronella additives in beef cattle, which was able to reduce the daily CH_4 production by 33 %. However, the utilization of CR as a component of ruminant diet has not been explored by pre-ingestive technology and information about its effect on rumen metabolism is scarce. Previous studies have shown that the application of 4 % ammonia and 6 % fermentation levels on different types of agricultural and plantation byproducts produced satisfying effects on rumen fermentation characteristics and the performance of animals [11-13].

This research was conducted to evaluate the effect of different pre-ingestive treatments to increase the nutritional potential of CR. Specifically, it aimed to determine the metabolic characteristics of fermentation and the formation of CH_4 from pre-ingestive CR under *in vitro* conditions. The information resulting from this study will be useful in increasing the proportion of local ingredients in ruminant diets in order to ensure the stability of ruminant feed supplies.

Materials and methods

Experimental diet preparations

The tropical grass used for the diet was napier grass (*Pennisetum purpureum*), which was trimmed from the pastures of the UPT Teaching Farm, Andalas University. The CR was collected from a citronella oil refinery unit in the Solok region, West Sumatra, Indonesia. The preparation of ammoniated CR began with chopping the CR (stems and leaves) to approximately 10 cm. The chopped CR was put inside a two-layered plastic bag to avoid leakage and was then compacted. The average dry matter (DM) content for the CR substrate used was 85 %, with a nitrogen dose of 4 % DM [11]. Furthermore, 10 kg of the substrate required 750 g of urea, which was dissolved in water by stirring until the solution was homogeneously mixed. The urea solution was poured into a plastic container and the chopped CR from the plastic bag was gently added into the urea solution. The plastic container was then sealed and stored in a warehouse for 3 weeks. The preparation of the fermented CR was carried out by sprinkling Starbio® (PT. LHM, Indonesia) at a dose of 6 % DM and urea at a dose of 0.6 % DM onto the compacted CR pile. The substrate was then stored in a sealed container for 10 days. At the end of the fermentation period, the CR was collected and each sample was dried for 24 h in an oven at 60 ºC. The sample was ground into powder (1-mm sieve) before it was proximately analyzed, and the *in vitro* rumen evaluation occurred. The composition of each treatment diet followed Elihasridas *et al.* [11] and was:

CR0: 100 % *P. purpureum* (control)
ACR1: 75 % *P. purpureum* + 25 % ammoniated CR
ACR2: 50 % *P. purpureum* + 50 % ammoniated CR
ACR3: 100 % ammoniated CR
FCR1: 75 % *P. purpureum* + 25 % fermented CR
FCR2: 50 % *P. purpureum* + 50 % fermented CR
FCR3: 100 % fermented CR

Proximate analysis

The determination of the nutritional value of the experimental diet, which consisted of DM, crude protein, crude fiber, crude fat, and ash, was based on the Association of Official Analytical Chemists [14] method (ISO/IEC 17025:2017). The ash content of the sample was determined using an electric furnace at a temperature of 550 °C for 6 h. The equation for the determination of ash content of the sample was: Ash weight/sample weight×100 %. The percentage of nitrogen-free extract (NFE) was calculated using the following equation: NFE = 100 % − (water + ash + crude protein + crude fat + crude fiber). The calculation of the total digestible nutrients was based on the formula of Sutardi [15]: 70.6 + (0.259×crude protein) + (1.01×crude fat) − (0.76×crude fiber) + (0.091×nitrogen-free extract). The analysis of the dietary fiber components, namely the neutral detergent fiber, acid detergent fiber, hemicellulose, cellulose, and lignin, was carried out according to the procedure of Goering and Van Soest [16].

In vitro rumen incubation procedure

A 250 mL Erlenmeyer flask (Duran Group GmbH, Germany) was used as an *in vitro* fermentation tube according to the method modified by Tilley and Terry [17]. The fluid intake of the rumen was used

as a microbial source and was collected from cattle bulls at public abattoir, Padang, Indonesia. A total of 2.5 g of feed samples were weighed and put into a fermenter, and artificial saliva (9.8 g sodium bicarbonate, 9.3 g disodium phosphate, $H_2O.12H_2O$, 0.57 g potassium chloride, 0.47 g sodium chloride, 0.06 g epsomite ($MgSO_4.7H_2O$), and 0.04 g calcium chloride dihydrate ($CaCl_2.2H_2O$) per liter of distilled water) was added. About 200 and 50 mL of rumen liquor was also added, and the mixture was stirred slowly. The addition of carbon dioxide was carried out for 30 s to form anaerobic conditions, and each tube was then sealed using rubber caps before being placed in a shaker bath. The temperature of the shaker bath was preconditioned at 39 °C for 24 h before the incubation stage began. The *in vitro* rumen fermentation activities were carried out for 48 h.

Determination of *in vitro* rumen fermentation characteristics

A digital pH meter (Hanna Instruments HI98107, USA) was used to measure the pH of the rumen fluid was carried out using a digital pH meter after calibration with standard pH solutions of 4.01 and 7.01 pH. Each fermentation tube was then immersed in iced water to stop further microbial fermentation. The rumen fluid was centrifuged at a speed of 4,000 rpm for 10 min. Then, 1 mL of the obtained supernatant was piped and dropped into a Conway cup filled with the chemical indicators of 1 mL sodium carbonate on the right side and 1 mL boric acid (2 %) in the center of the cup to obtain the NH_3-N concentration following the microdiffusion method [18]. The partial and total volatile fatty acid (VFA) components were determined by placing 2 mL of the rumen fluid into a fermentation tube and then transferring it to a 10 mL tube before adding 30 mg of sulfosalicylic acid. The mixture was then homogenized using an automatic stirrer to obtain the protein deposits. Furthermore, the solution was centrifuged at a speed of 3,000 rpm for 15 min to obtain the supernatant. Then, the supernatant was filtered using a millipore filter (0.22 nm). About 1.0 µL of the filter product in the form of a clear solution was then injected into a gas chromatography (GC) apparatus (Varian 3700, USA) with a capillary column length of 30 m, equipped with pure nitrogen flow as a carrier gas. Fatty acid separation was detected by a flame ionized detector, and the identification and quantification of the partial VFAs were performed using the standard carbon$_2$ (C_2)–C_5. (WSFA-2, Sigma-Aldrich, Inc., USA).

The procedure for determining microbial protein synthesis was adopted from Pazla *et al.* [19] using centrifugation and spectrophotometric procedures (UV-visible U-1800, High-Technologies, Japan). The calculation of the protozoa population was based on Ogimoto and Imai [20] by staining the rumen fluid sample with a solution consisting of 100 mL formaldehyde, 0.6 g formalin saline, and 9 g NaCl dissolved in 900 mL water. The counting stage used a counting chamber with the rumen fluid sample that had been diluted 5 times with a saline-distilled formalin solution. The calculation of the number of protozoan cells was carried out by observing 5 boxes (0.1 mm^3/segment) that had rumen fluid dripped into the counting chamber. The observations were performed using a microscope with 40 - 100× magnification. The protozoa population per mL of rumen fluid was calculated using this equation: protozoa population = (n/5×104×DF), where n = number of protozoa and DF = dilution factor. The estimation of the rumen CH_4 emissions was carried out using the formula from Moss *et al.* [21]: CH_4 emission (% total VFA) = $(0.45\times C_2) - (0.275\times C_3) + (0.40\times C_4)$.

Statistical analysis

The data on rumen metabolism and CH_4 production were analyzed to determine the variance between treatments according to statistical model: $Yij = \mu + \tau i + \varepsilon ij$, where Yij are the rumen fermentation variables, µ is the average mean, τi is the additive effect of the prior treatment, and εij is the experiment error. Data integration was performed using JASP 0.13 software [22]. If the treatment was deemed significant at a *p*-value of $p < 0.05$ or < 0.01, the post-hoc Tukey HSD test was performed.

Results and discussion

Effect of pre-ingestive treatment on the nutritional values of citronella residues

The analysis of nutritional values of the experimental diets (**Table 1**) showed that the DM content ranges from 88.55 - 93.11 %. The best CP contents in a row were FCR1 (12.04 %), CR0 (11.97 %), and ACR1 (11.72 %). The crude fiber content increased in ACR3 by 10 % and FCR3 by 36 % compared with CR0. Meanwhile, the highest crude fat contents were in the diets composed of 100 % CR: FCR3 (2.39 %) and ACR3 (2.03 %). The CR fermentation treatment provided more ash content than the other treatments. The ACR2 and ACR1 treatments had NFE content values that were similar to the control, namely 45.09 and 44.97, respectively. Meanwhile, the ammonia treatments showed a greater proportion of TDN. The crude fiber fractions, namely NDF and ADF, were lowest in FCR1 (68.57 and 31.90 %, respectively). An

increase in the ratio of CR to *P. purpureum* resulted in lower hemicellulose levels. Conversely, the cellulose and lignin content tended to increase with increasing CR.

Chemically or biologically treating CR showed promising improvements to its nutritional profile as a source of fiber feed for tropical ruminants. Manurung *et al.* [23] reported that the crude protein content in *Cymbopogon* residue (without pre-ingestion) was at 9.72 % DM. A comparative study of the nutritive value of the residue and fresh material of *C. nardus* by Sari *et al.* [5] showed lower crude protein (5.82 % versus 7.15 % for residue and fresh material, respectively) and NDF (73.67 % versus 70.17 %, respectively) compared with the results of the current study. Furthermore, the analysis of agricultural waste (palm frond oil) as a basal diet showed a crude protein composition level of 4.35%, NDF of 74.06 %, and ADF of 51.72 % [24]. The fungal treatment to improve the nutritional quality of the palm frond provided a cellulose level of 33.26 % and a hemicellulose level of 14.41 % [25]. This indicates an inferior nutritional profile of palm fronds when compared with ACR3 and FCR3 (100 % citronella residue treatments). The chemical profile of agricultural and plantation waste in tropical areas is identical to their high structural carbohydrate (lignocellulose) contents and low essential macro nutrient contents, such as protein and minerals, which are factors limiting the use of these as feed.

The application of pre-ingestive technology on these agriculture byproducts that are high in lignocellulose can stretch the structure and cell walls of the plants and facilitate the penetration of extracellular enzymes from the rumen microbiome. In the fermentation treatment, an increase in the crude fat content was associated with a reduction in the complex carbohydrates content [26]. The presence of lactate bacteria can produce fibrolytic enzymes during fermentation that can breakdown the oligosaccharide structure, improving nutrient bioavailability in the CR. The application of feed technology (such as ammonia and fermentation) on CR, which is more reliable to apply in smallholder farmer, is a promising solution to improve the nutritional quality and increase the consumption levels as well as increase the pre-ingestive CR to about 3.74 % compared with the control.

Table 1 Nutritional value of experimental diets [11].

Nutritional value (% DM)	Treatment							S.E.M.
	CR0	ACR1	ACR2	ACR3	FCR1	FCR2	FCR3	
Dry matter	93.11	92.32	91.77	89.97	92.10	90.70	88.55	0.594
Crude protein	11.97	11.72	11.26	10.88	12.04	10.55	9.76	0.315
Crude fibre	25.14	25.77	26.41	27.67	27.42	29.70	34.25	1.170
Crude fat	0.87	1.16	1.45	2.03	1.25	1.63	2.39	0.199
Ash	9.93	8.70	7.57	9.37	9.71	10.18	10.57	0.384
Nitrogen-free extract	45.2	44.97	45.09	40.02	41.68	38.65	31.58	0.380
Total digestible nutrient	50.76	51.52	52.29	53.81	50.02	49.27	47.78	0.754
Neutral detergent fiber	71.07	71.92	72.20	70.92	68.57	67.28	64.71	1.051
Acid detergent fiber	35.02	36.22	36.17	38.66	31.90	37.17	39.22	0.924
Hemicellulose	36.05	35.70	36.03	32.27	36.67	30.12	25.49	1.575
Cellulose	31.66	32.84	30.95	33.17	27.40	30.86	30.14	0.728
Lignin	2.80	2.87	5.01	4.82	4.29	4.94	7.65	0.616

CR0: 100 % napier grass; ACR1: 75 % napier grass + 25 % ammoniated CR; ACR2: 50 % napier grass + 50 % ammoniated CR; ACR3: 100 % ammoniated CR; FCR1: 75 % napier grass + 25 % fermented CR; FCR2: 50 % napier grass + 50 % fermented CR; FCR3: 100 % fermented CR. S.E.M.: standard error of the mean.

Effect of pre-ingestive citronella residue on *in vitro* rumen fermentation metabolism

The rumen fermentation characteristics, studied using an *in vitro* method, showed highly significant differences in the pH of the rumen liquid ($p < 0.001$), which ranged from 6.68 - 6.77 (**Table 2**). The ACR1 treatment produced 7.74 mM NH_3-N, which was not significantly different from CR0 ($p > 0.05$) but was significantly different than the other treatments ($p < 0.001$). The effect of replacing *P. purpureum* with pre-ingestive CR did not result in a significant Change in the total VFA. An increase in the ratio of

pre-ingestive CR to *P. purpureum* contributed to a significant decrease in the total molar accumulation of iso-VFA ($p < 0.001$). Regarding the partial VFA, the pre-ingestive CR had a significant effect on composition of the total VFA by affecting the percentage of acetic acid, propionic acid, n-butyric acid, and iso-valeric acid ($p < 0.05$). Pre-ingestive CR also significantly affected the percentage of iso-butyric acid, n-valeric acid, and the ratios of C_2:C_3 and (C_2 + C_4):C_3. **Figure 1** shows that the treatments with 25 % CR, namely ACR1 and FCR1, resulted in microbial protein synthesis rates of 108.99 mg/100 mL and 116.43 mg/100 mL, respectively, which were almost identical to CR0 with a value of 117.14 mg/100 mL and not significantly different ($p > 0.05$). In contrast, **Figure 2** shows that an increase in the CR ratio significantly reduced the rumen protozoa population ($\times 10^5$) by 39.17 % compared with CR0 ($p < 0.01$). **Figure 3** presents the effect of the experimental diet on the estimated *in vitro* rumen CH_4 production (% total VFA), which was not significantly different among treatments ($p > 0.05$). However, there was a slight reduction in CH_4 production.

The pH of the rumen fluid during the incubation period was relatively stable. In line with the *in vitro* fermentation pattern, Fitri *et al.* [27] reported that when using persimmon peel substrate (0 - 150 g/kg DM) as feed ingredients in the total mixed ration, the pH ranged from 6.34 - 6.37 and the NH_3 levels were 6.39 - 7.37 mg/dL. The total VFA production, which resulted from substituting various levels of *C. nardus* residue, was lower than that recorded in previous studies. From an *in vitro* evaluation of 100 % *Cymbopogon winterianus* waste, Manurung *et al.* [23] reported a VFA production rate at 187.57 mM, which strongly contrasts with ACR3 (37.55 mM) and FCR3 (34.79 mM) from the current study. It can be understood that *C. winterianus* is a superior citronella species compared with *C. nardus*, where crude fiber content was 5 - 12 % higher. Structural carbohydrates in rumen fiber fermentation reduce VFA biosynthesis.

Table 2 *In vitro* rumen fermentation characteristics.

Item	Treatment							S.E.M.	*p*-value
	CR0	ACR1	ACR2	ACR3	FCR1	FCR2	FCR3		
pH	6.68^{a}	6.72^{a}	6.73^{a}	6.77^{b}	6.68^{a}	6.73^{a}	6.76^{b}	0.012	< 0.001
NH_3-N[A]	7.98^{a}	7.74^{ab}	7.26^{b}	6.22^{c}	6.87^{bc}	6.60^{c}	6.45^{c}	0.103	< 0.001
Total VFA[A]	40.74	39.36	34.84	34.50	37.55	34.79	34.38	3.596	0.427
Total iso-VFA[A]	2.67^{b}	2.47^{b}	2.16^{ab}	1.71^{a}	2.14^{ab}	2.56^{b}	1.80^{a}	0.180	< 0.001
Acetic acid (C_2)[B]	68.97^{a}	67.13^{ab}	66.08^{b}	66.21^{ab}	66.70^{ab}	65.33^{b}	67.12^{ab}	0.844	0.020
Propionic acid (C_3)[B]	15.30^{ab}	14.80^{ab}	15.05^{ab}	15.47^{ab}	14.84^{ab}	14.17^{b}	16.48^{a}	0.572	0.036
iso-Butyric acid ($_iC_4$)[B]	2.25	3.01	2.74	2.62	2.29	3.36	2.51	0.351	0.069
n-Butyric acid ($_nC_4$)[B]	9.59^{a}	10.09^{ab}	11.33^{ab}	10.64^{ab}	11.39^{ab}	11.53^{b}	9.63^{a}	0.615	0.020
iso-Valeric acid ($_iC_5$)[B]	2.74^{a}	3.58^{ab}	3.46^{ab}	3.67^{b}	3.42^{ab}	4.08^{b}	2.77^{a}	0.291	0.004
n-Valeric acid ($_nC_5$)[B]	1.15	1.39	1.33	1.38	1.36	1.53	1.5	0.112	0.080
C_2:C_3	4.52	4.54	4.41	4.28	4.5	4.62	4.08	0.198	0.189
(C_2 + C_4):C_3	5.14	5.21	5.16	4.97	5.26	5.43	4.66	0.218	0.071

A as mM, B as % total VFA

CR0: 100 % napier grass; ACR1: 75 % napier grass + 25 % ammoniated CR; ACR2: 50 % napier grass + 50 % ammoniated CR; ACR3: 100 % ammoniated CR; FCR1: 75 % napier grass + 25 % fermented CR; FCR2: 50 % napier grass + 50 % fermented CR; FCR3: 100 % fermented CR. S.E.M.: Standard error of the mean. Different superscripts (a, b) in a row showed different significance level.

The VFA concentration in the feed ration containing pre-ingestive CR was slightly different to other treated tropical agricultural byproducts. Dewi *et al.* [28] reported that ammoniated cocoa pods and coffee husks (5 % urea) using fiber cracking technology (135 °C; 2.3 atm) for 2.5 h had a molar VFA production rate of 39 - 44 mM. The molar percentages of acetic acid and n-butyric acid from the current experiment were higher than those of Bureenok *et al.* [29], who used *Pennisetum* silage (without additives), whereas the propionic acid concentration was slightly lower. Furthermore, Wanapat *et al.* [30] reported that the

fermentation characteristics of Brahman native cattle supplemented with lemongrass powder (0 - 300 g/d) showed ratios of C_2:C_3 and (C_2 + C_4): C_3 that were between 2.8 - 4.0. This indicates the potential of CR as a fiber source to replace *P. purpureum* in terms of improving the rumen fermentability profile.

However, the utilization of CR as a basal diet requires the inclusion of quality protein sources to improve the rumen fermentation characteristics. Previous research has shown that supplementing ammoniated rice straw-based total mixed ration with 10 - 30 % tropical legumes (i.e., *Gliricidia sepium* and *Leucaena leucocephala*) resulted in higher NH_3-N production, total VFAs, acetate:propionate ratio, and digestibility when compared with the control [31,32]. In addition, Chumpawadee *et al.* [33] reported that an increase in the *in vitro* DM digestibility value by over 50 % can be achieved through protein feed sources, namely soybean meal (60.96 %) and peanut meal (52.02 %).

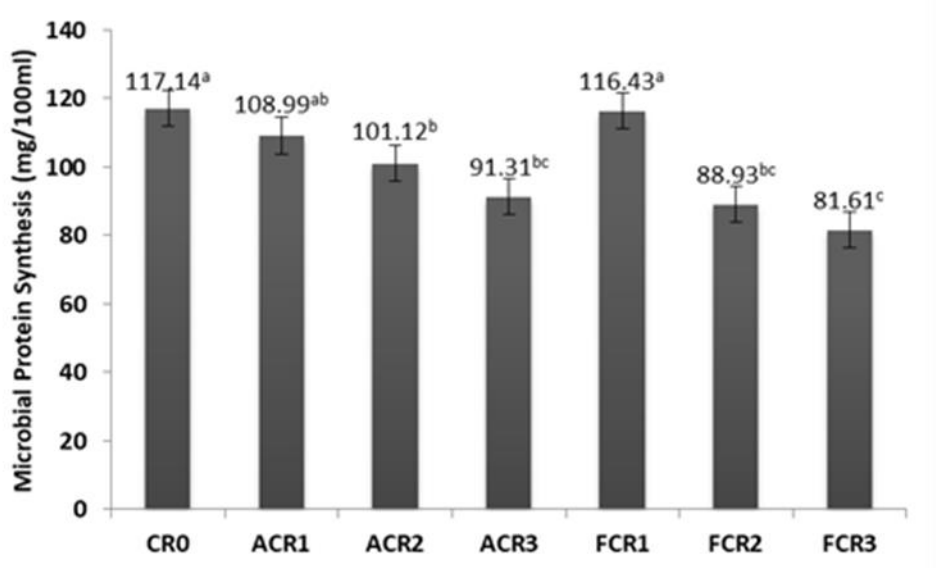

Figure 1 Microbial protein synthesis (mg/100 mL) of experimental diets. CR0: 100 % napier grass; ACR1: 75 % napier grass + 25 % ammoniated CR; ACR2: 50 % napier grass + 50 % ammoniated CR; ACR3: 100 % ammoniated CR; FCR1: 75 % napier grass + 25 % fermented CR; FCR2: 50 % napier grass + 50 % fermented CR; FCR3: 100 % fermented CR. Different superscripts (a, b, c) in a row showed higher significance level ($p < 0.01$).

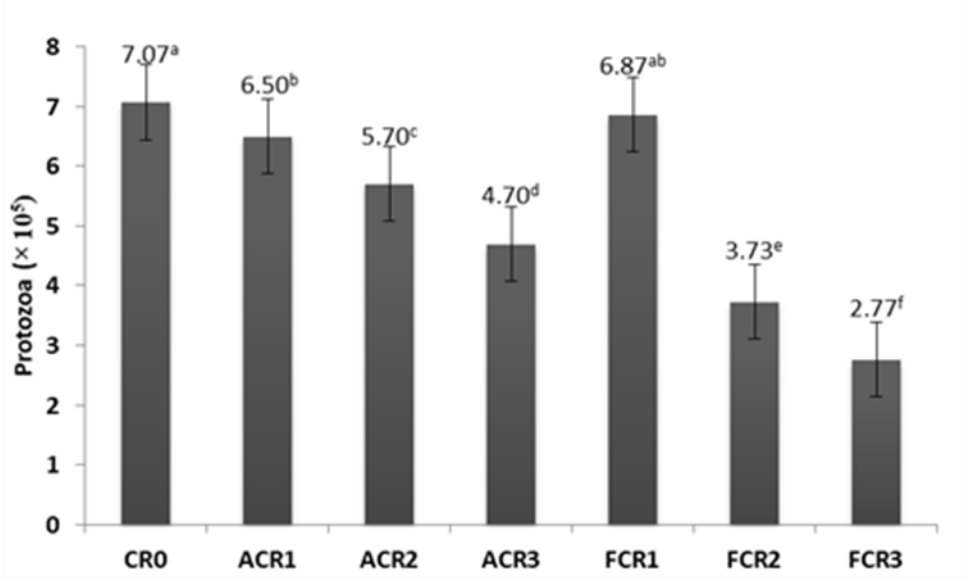

Figure 2 Protozoa population ($\times 10^5$) of experimental diets. CR0: 100 % napier grass; ACR1: 75 % napier grass + 25 % ammoniated CR; ACR2: 50 % napier grass + 50 % ammoniated CR; ACR3: 100 %

ammoniated CR; FCR1: 75 % napier grass + 25 % fermented CR; FCR2: 50 % napier grass + 50 % fermented CR; FCR3: 100 % fermented CR. Different superscripts (a, b, c, d, e, f) in a row showed higher significance level ($p < 0.01$).

The use of pre-ingestive CR at various substitution levels favored microbial protein synthesis. This was proven through the *in vitro* evaluation of 30 % fermented straw-based feed, with 30 % *P. purpureum* showing a microbial protein concentration of 71.50 mg/100 mL [34]. Furthermore, lower results were reported by Widyawati *et al.* [35], who used a mixture of protein supplements (namely soybean meal-*Cassia alata*) and measured a production level of 2.93 mg/100 mL. The crude protein content of the diet greatly influenced the level of microbial protein synthesis. More specifically, a proportional balance of VFA components accompanied by sufficient levels of NH_3-N (> 3.6 mM) is a driving factor for rumen microbial protein biosynthesis [36]. The effect of *Cymbopogon citratus* meal (100 g/d) supplementation on beef cattle formed a protozoa population of 5.7×10^5 and estimated CH_4 production at 28.8 mL/100 mL [9], whereas pre-ingestive CR resulted in a lower but almost equivalent microfauna profile in rumen CH_4 production. In addition, Elihasridas *et al.* [11] showed *in vitro* DM and organic matter digestibility from pre-ingestive CR ranged from 42.62 - 43.72 and 41.35 - 42.69 %, respectively [11].

The decrease in the protozoa population, which was associated with an increase in the CR level, was made possible by the low availability of fermentable carbohydrates in order to satisfy the metabolic activity of the protozoa. There was also evidence that the CR still contained phytochemicals in the form of volatile terpenoids, i.e., linalool and citronellal, that are said to have antiprotozoal bioactivity [37,38]. More recently, it was reported that CR was rife with phytochemicals such as condensed tannins (gallic acid), flavonoids, phenolic acids (caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid), and essential oils [6]. We acknowledge our recent observations did not involve determining these compounds. However, Purba *et al*. [39]; Purba *et al*. [40] reported that the combination of the plant matrix with such compounds (including gallic acid, flavonoid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, and essential oils) consistently resulted in increasing fermentable ruminal carbohydrates and reducing protozoa. However, Purba *et al*. [41] reported that among the polyphenols, essential oils have a greater effect on ruminal fermentation. Supplementation with essential oils alone did not change the effects on fermentable carbohydrates but did, however, reduce the number of protozoa. Thus, it indicates that the phytochemical content of CR might influence *in vitro* ruminal fermentation and affect the protozoa population.

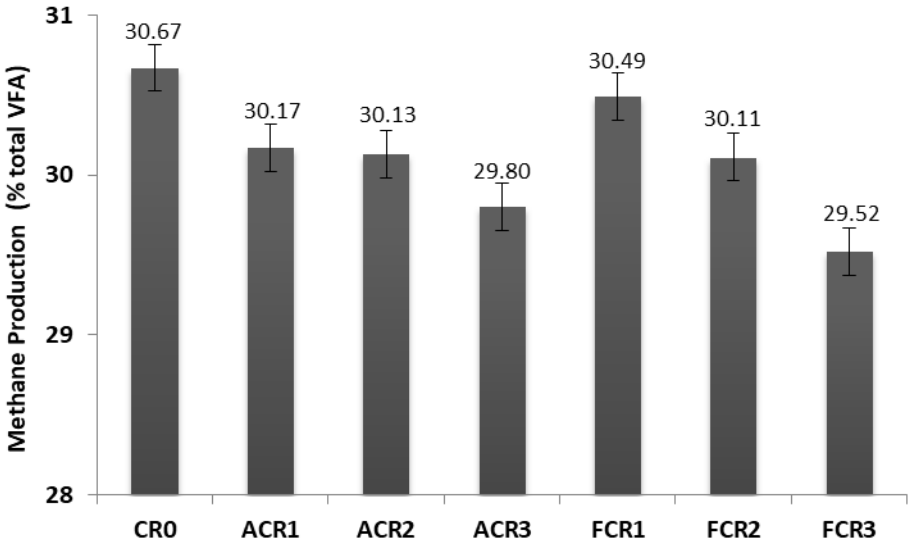

Figure 3 Methane production (% total VFA) of experimental diet. CR0: 100 % napier grass; ACR1: 75 % napier grass + 25 % ammoniated CR; ACR2: 50 % napier grass + 50 % ammoniated CR; ACR3: 100 % ammoniated CR; FCR1: 75 % napier grass + 25 % fermented CR; FCR2: 50 % napier grass + 50 % fermented CR; FCR3: 100 % fermented CR.

Cymbopogon citratus showed more promising results, where a supplementation level of about 4 % DM in beef steers reduced CH_4 production by 33 % and increased the average daily gain to 1.20 kg/d

[10]. There are indications that the product of plant material biodegradation (in form of acetate) is the primary source of CH_4 production. This is because the consumption of a high fiber diet by ruminants stimulates the development of a cellulolytic bacterial population with predominant acetate production. Acetic acid becomes the main substrate for *Methanobacterium* and Archaea in rumen methanogenesis [42]. However, the CH_4 profile was lower than when *Hibiscus rosa-sinensis* leaves and *Sapindus rarak* fruits with saponin levels of 16.6 - 36.4 % DM were used, which had methane production levels of 36.8 and 35.1 % of total VFAs, respectively [43]. Suriyapha *et al.* [44] reported that bamboo grass (*Tiliacora triandra*) pellet supplements used as a rumen modifier at 150 g/head/d produced CH_4 ranging from 26.0 - 30.5 CH_4 mmol/100 mol.

Furthermore, *in vitro* rumen CH_4 production profiles in various agro-industrial byproduct variants were reported by Samadi *et al.* [45] and show ketchup residues had the lowest level (33.88 mL/g DM), while rice bran the highest (35.18 mL/g DM). This proves that the application of a pre-ingestive on CR can result in lower or equivalent CH_4 production profile compared with other feed formulations. It is expected that the use of pre-ingestive CR as a component of ruminant feed rations can reduce the effects of environmental pollution and increase the energy efficiency of rumen fermentation.

Conclusions

The utilization of the pre-ingestive methods of ammoniation and fermentation on CR as an alternative basal diet for ruminants showed equal VFA profiles and rumen CH_4 production at various levels. However, an increase in the levels of pre-ingestive CR resulted in lower microbial protein synthesis (16 %) and protozoa population (29 %) compared with *P. purpureum* alone. Further studies will be focused on screening the phytochemical content of CR after various pre-ingestive technologies (i.e., steam, acid, and mechanical treatment) with larger sample sizes and their combination with a protein-source feed to improve the rumen fermentation profile.

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