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Ruminal volatile fatty acid concentration and methane gas production in sheep (*Ovis aries*) fed two types of basal diets with or without *Gliricidia sepium* legume forage supplementation

R-Jun Frederick A. Gaspe[1,2,3*], Lolito C. Bestil[2] and Taketo Obitsu[1]

ABSTRACT

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The aim of this study was to clarify whether the type of forage diets Napier grass (*Pennisetum purpureum*) vs. urea-treated rice straw and supplementation with *Gliricidia sepium* affect rumen VFA production and methane emission as well as intake and digestibility in ruminants. The experiment was set up in a randomized complete block design (RCBD) composed of four treatments namely: Napier grass (T1), Napier + legume forage (T2), urea-treated rice straw (T3), and urea-treated rice straw + legume forage (T4). Each treatment was divided into four blocks (n=16) based on body weight and sex combination.

The daily dry matter intake tended to increase from treatments supplemented with *G. sepium* (T2 and T4) compared to treatment-fed pure basal diet alone (T1 and T3). Intake relative to metabolic weight ($BW^{0.75}$) is high in T2 and T4. The molar concentration of volatile fatty acid was higher in T1, which was found comparable with T2 and T3, while T4 has the lowest. The molar proportion of acetate tended to increase in T1 and T3, while the propionate concentration was significantly high in T2 and T4 leading to lesser CH_4/CO_2 production respectively. These results confirmed the potential of *G. sepium* as a methane-mitigating supplement to basal diets such as Napier and urea-treated rice straw as feed for ruminants.

Keywords: climate change, methane mitigation, legume forage, VFA, CH4 production

[1]Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima, 739-8528, Japan
[2]Department of Animal Science, Visayas State University, Baybay City, Leyte 6521- A
[3]Agriculture Department, Capiz State University, Pilar Satellite College, Natividad Pilar, Capiz 5804

***Corresponding Author.** Address: Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima, 739-8528, Japan; Email: rfagaspe@capsu.edu.ph

INTRODUCTION

The increasing atmospheric concentration of greenhouse gases (GHGs) such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (NO_2), resulting in climate change is a major environmental, economic, and social threat worldwide (Beauchemin et al 2009). Increasing global temperature, changes in precipitation patterns, extreme weather events, and an increase in sea level are events associated with climate change (PCARRD 2009). In tropical countries like the Philippines, where agriculture is the main source of living, the impact of this climate change is immense. The 2017 World Risk Report ranks the Philippines as the third most vulnerable nation to climate change due to its high exposure to natural hazards (cyclones, landslides, floods, droughts), dependence on climate-sensitive natural resources, and vast coastlines where all of its major cities and the majority of the population reside (NICCDIES 2021). Agriculture is a major source of greenhouse gas (GHG) emissions and is also susceptible to climate change (OECD Meeting of Agriculture Ministers 2022). The sector currently generates approximately 19-29% of the total global anthropogenic GHG emissions, and fifty percent of these are in the form of methane and nitrous oxide (Climate-smart agriculture, World Bank 2021). Philippine agriculture is made up of four sub-sectors: farming, fisheries, livestock, and forestry (Statistica Research Department 2022). Overall, livestock contributes approximately 40% of anthropogenic GHG emissions (Sun et al 2023), two-thirds of which are from the ruminant sector (Cardoso-Gutierrez et al 2021). Livestock emit GHGs directly from enteric and manure fermentation or indirectly from feed-production activities and forest conversion into pasture (Gerber et al 2013). Enteric CH_4 from ruminant production systems has been reported as the largest source of GHG (Martin et al 2010), with a global warming potential (GWP) of 25 times compared to CO_2 (Haque 2018). In the Philippines, ruminants are widely raised in commercial and backyard farms as a source of food and income. As the ruminant population is continuously increasing, an increase in methane emission is also expected.

The structure of the digestive system of ruminants harbors a large number of microbes that can convert feeds rich in fiber, which is non-valuable from a human perspective, into highly valuable products such as milk and meat (Immig 1996). In that sense, ruminants are important since they do not compete with humans for the same food (Moss et al 2000). This unique feature of ruminants, however, yields CH_4 and CO_2 as natural products of microbial fermentation and to a lesser extent, amino acids (AA) in the rumen and hindgut of farm animals. During the oxidative process under anaerobic conditions, the glucose in plant polymers and starch is fermented to pyruvate and lactate (Moss et al 2000). This gives NADH that is then re-oxidized to NAD to complete the fermentation of sugars. By transfer of electrons to acceptors other than oxygen, the NAD+ is regenerated. Propionate plays a crucial role in the energy metabolism of ruminants, serving as a primary precursor to glucose. Its production in the rumen not only provides an alternative avenue for hydrogen (H) disposal but also contributes to a substantial reduction in methane production. There are three main pathways through which ruminal propionate is formed: the succinate, acrylate, and propanediol pathways. Among these, the succinate and acrylate pathways are the primary contributors to propionate production and are effective in disposing of excess hydrogen. Specifically, when propionate is generated from succinate, it results in the production of carbon dioxide. This interaction between propionate and carbon dioxide, coupled with the free hydrogen

(H2) generated during NADH re-oxidation, ultimately leads to the formation of water and methane. Redirecting hydrogen (H) from methane production to propionate formation holds great potential as a strategy for reducing methane emissions, given the positive effects of propionate on ruminants (Wang et al 2023).

With the need to increase ruminant production in response to the increasing human population's need for meat and milk, abatement strategies are necessary to reduce GHG emissions from this sector. In the last decades, several nutritional abatement solutions with various approaches to mitigate CH_4 emissions have been evaluated; each has its own set of benefits and drawbacks (Danielsson 2016).

The inclusion of legume foliage and pods in the ration as a cheap protein source is a potential alternative to reduce greenhouse gas emissions in the tropics. These forage species usually contain condensed tannins and saponins that alter the rumen microbes such as archaea, protozoa, and fibrolytic bacteria, affecting fermentative processes such as reduced fiber digestibility, while increasing protein and energy supply available to the animal (Molina-Botero et al 2019). Thus, supplementing poor-quality roughage with local forage sources of fresh legumes like *Gliricidia sepium* is believed not only to practically improve feed intake, nutrient digestibility, and ruminant production but also to mitigate enteric CH_4 emissions. A variety of forage legumes were tested for their potential as defaunating agents (Aban & Bestil 2016) but not for their effect on methane production. To our knowledge, there is a lack of in vivo studies on enteric CH_4 mitigation from ruminants in the Philippines using feed sources like *Pennisetum purpureum* (Napier grass) and urea-treated rice straw (UTRS). This study aimed to clarify whether *Gliricidia sepium* locally known as *Kakawate,* supplementation and the kind of forage diet (Napier vs. urea-treated rice straw) affect rumen VFA production and methane emissions from sheep, as well as intake and digestibility.

MATERIALS AND METHODS

Animals, Dietary Treatments, and Experimental Design

Animal care and experiments were conducted according to the guidelines of the Animal Care and Use Committee of the Department of Animal Science, Visayas State University, Baybay City Leyte.

Sixteen merino sheep (*Ovis aries*) male (n=9), female (n=7), aged 6-7 months (mean BW 16.49kg ± 8.40 SE), were used in this study, following a randomized complete block design (RCBD) with a 2x2 factorial arrangement, with factor 1 as the type of forage diet and factor 2 as the supplementation with *G. sepium* legume forage. Differences in sex and body weight of the sheep were used as a basis for blocking. The sheep were dewormed one week before the start of the study using Ivermectin administered subcutaneously at 0.03mL kg^{-1} body weight to ensure that the sheep were free of parasites at the start of the study. The experimental area and metabolism cages were disinfected with a multipurpose disinfectant one week before the start of the experiment. The sheep were confined for one month, with a two-week adjustment period, in metabolism cages measuring 1.5m in length, 0.75m wide, and 1.5m in height, with an excrement separator.

Four dietary treatment combinations were tested namely: Napier grass (*Pennisetum purpureum)* without supplement (T1) and with kakawate *(G. sepium)* legume forage supplement (T2); and UTRS without supplement (T3) and with

kakawate legume forage supplement (T4). Each treatment was replicated four times.

Preparation of Forages

Napier grass, aged 45-60 days after cutting, was gathered early in the morning from the pasture area of the Small Ruminant Project at the Visayas State University. The Napier grass soilage was chopped to a length of 3-5cm long for easy utilization by the animals and served as the basal forage for T1 and T2.

Urea Treated Rice Straw

Rice straw was chopped to a particle size of 3 to 5 cm before treatment with urea. Ten kg of rice straw (on a dry matter [DM] basis) was spread on a clean, sanitized concrete floor. Then, 0.5kg commercial urea was dissolved in 10L of water and the urea solution sprayed throughout the chopped straw using a knapsack sprayer. The straw was mixed thoroughly to achieve uniform wetting with the urea solution. The treated straws were then incubated for 3 days by covering the treated material with canvas and then dried for half a day to attain at least 86% DM for longer storage. This UTRS was provided for treatments T3 and T4.

Kakawate (*G. sepium*), a legume forage dominantly available at the locality, was collected and mixed with Napier grass soilage and UTRS at 30% (Tomkins et al 1991) of the total ration on a DM basis for treatments T2 and T4, respectively.

Table 1.0 Chemical composition of experimental diets.

Parameter	Feed source		
	Napier	Urea-Treated Rice Straw (UTRS)	Kakawate (*Gliricidia sepium)*
DM (%)	41.06	86.14	31.32
CP (%)	9.7	7.9	15.83
GE (MJ kg^{-1} DM)	17.4	16.0	18.41
NDF (%)	74.31	85.15	45.81
Lignin	5.7	52.0	13.0
Soluble Tannins (g k^{-1} DM)	22.2	-	146.5
Condensed Tannin (g kg^{-1} DM)	12.4	-	15.34

DM (%) = percent dry matter; CP (%)=crude protein in percent; GE (MJ kg^{-1} DM)=gross energy in megajoules per kilogram of dry matter; NDF (%)=neutral detergent fiber in percent; lignin and tannin content of Napier and UTRS were obtained from the feedipedia website (https://www.feedipedia.org/), while that of the legume supplement was obtained from the study of Oden et al (2000)

Feeding Procedure

The experimental diets were fed twice a day, at 8am and 5pm. Water was available for the animals throughout the study.

During the adjustment period, sheep were provided experimental diets *ad libitum* from day 1 to day 8 with an additional 20% allowance of the day's offering based on the previous day's intake. From days 9 to 21, sheep were provided with each experimental diet at a fixed amount which was equivalent to the least DM

intake among diets determined from day 1 to day 7. From days 17 to 21, samples of diets, rumen fluid, feces, and expired gas around the mouth of sheep were collected.

Sample Collection and Measurements

Diets and feces

Samples of diets and refusals were collected during the last 5 consecutive days of the experimental period. Daily fecal output was also determined and sampled in the last 5 consecutive days. The daily fecal samples were then pooled for each animal and a sub-sample was used for later analysis on dry matter and NDF digestibility.

Dry matter contents in diets, refusals, and feces were measured by drying the samples at 100^0C for 24h. To determine the content of neutral detergent fiber (NDF_{om}) including ash, the diets, refusals, and feces were dried at 60^0C, then ground using a Willey mill and passed through a 2mm screen. Determination was made by weighing 1g of the sample into a porcelain crucible, 100mL of neutral detergent fiber (NDF) solution was added, and 10mL of n-octanol. Then, it was heated to boiling and refluxed for 60mins using a Labconco Fiber Extractor (Expotech USA, Houston Texas), filtered and washed 3 times with boiling water, and dipped in acetone. The crucibles were then dried for 8h at 105^0C, contents reduced to ash in a muffle furnace at 600^0C for 2h, cooled down in the desiccator, and weighed (Van Soest 1963).

Rumen Fluid Samples

Rumen fluid was collected, via a stomach tube inserted through the mouth, once a day at the following times: In the morning 1h before feeding and 0.5, 2, 6, and 8h after feeding. To avoid the stress of the insertion of the stomach tube, the collection was conducted at a one-time point per day for 5 days. A 25mL syringe was attached to the stomach tube to collect the rumen fluid by suction. The pH of the rumen fluid was measured using the digital pH meter with a glass electrode (Milwaukee pH hand meter SM101, Northern California) right after collection. After pH measurement, the rumen fluid was filtered with a 2-4-layer cheesecloth. Filtered 30mL rumen fluid was further mixed with 1.2mL of 25% (w/v) sulfuric acid aqueous solution to inhibit further microbial fermentation. The rumen fluid was placed in sample bottles and stored in a refrigerator ready for volatile fatty acid (VFA) analysis.

Ten mL of rumen fluid was put into a Kjeldahl flask and water added to 300mL volume, then a further 10mL of 20% (w/w) magnesium sulfate aqueous solution was added, and 5mL of 50% (w/w) sulfuric acid aqueous solution for acidification. Then the mixed solution was distilled by steam and 150-200mL of distillate was collected in an Erlenmeyer flask. The distillate was then titrated with 0.1mol L^{-1} sodium hydroxide solution to measure the concentration of total VFA (Kromann et al 1967, Ibáñez et al 2014).

After titration, 0.5mL of 0.1mol sodium hydroxide was added to alkalize the distillate. For VFA composition analysis, 1.0mL of the alkalized distillate was mixed with 0.05mL of 25% (w/w) meta-phosphoric acid, and 0.5mL of internal standard (3mmol L^{-1} 4-methyl valeric acids). Before analysis, three levels of pure VFA

standards were prepared for calibration. Individually weighed solid or liquid reagents of pure sodium acetate (2.40g), sodium propionate (1.92g), n-sodium butyrate (2.20g), iso-butyric acid (0.35g), n-valeric acid (0.40g), and iso-valeric acid (0.40g) were transferred into separate flasks. Each reagent was then subsequently mixed with 100mL of distilled water. Additionally, the exact amount of 10mL for acetate and propionate, 4mL for n-sodium butyrate, and 5mL for butyric and valeric acid were dispensed into respective 50mL containers, distilled water was then added to fill to the 50mL mark. The various standards were then placed into plastic bottles and stored in the refrigerator. A 4-methyl valeric acid (MW 116.16) internal standard was prepared by weighing 0.348g of the liquid reagent into a 100mL volumetric flask and filling it to the mark with distilled water. Subsequently, 10mL of the solution was diluted to 100mL, placed in a plastic bottle, and stored in the refrigerator. Three standard solutions were prepared by taking 1mL of previously prepared standards and adding 0.2mL of metaphosphoric acid. Subsequently, an amount of 0.05mL, 0.10mL, and 0.20mL of the resulting standard with metaphosphate were separately placed into individual Eppendorf tubes and then mixed with 0.5mL of the internal standard. Distilled water was then added in quantities of 0.95mL, 0.90mL, and 0.80mL, respectively, to each standard. The solutions were thoroughly mixed and transferred into individual GC vials for further analysis (Erwin et al 1961).

This mixture was analyzed for the individual VFA with gas chromatography with a flame-ionized detector (GC17A, Shimadzu, Kyoto, Japan). A BP-21 column (30m length x 0.53mm diameter, 0.5µm in thickness, (SGE Analytical Science Australia) was used.

Collection and Analysis of Respiration Gas

On the final day of the experimental period, gas around the mouth of the sheep was collected at several time intervals (1h before feeding, 0.5, 1, 2, 4, 6, and 8h after feeding). This was done by covering the head of the sheep with a plastic bag 14 inches x 22 inches in size, attached to a 12mL syringe. To avoid suffocation of the animal, the air collection was done by sucking for about 0.5min after attaching the bag to the head. A portion of the air sample (10mL) was injected with a syringe into an evacuated sealed tube. The concentration of CH_4 and CO_2 in the air sample was measured by gas chromatography (Shimadzu GC-8A) with a TCD detector. The ratio of the peak area of CH_4 to CO_2 recorded on the chromatogram was used as an indicator of Ch_4 production. The ratios were averaged for each sheep per treatment.

Statistical Analysis

Data from five sampling days for each treatment were subjected to a two-way analysis of variance (ANOVA) using JMP Software (SAS, Institute Inc.). Significance was declared at $p<0.05$ and a tendency was considered up to $p<0.15$. A Tukey post hoc analysis was performed when variations among treatment mean were found to be significant.

RESULTS AND DISCUSSION

The chemical composition of Napier grass, urea-treated rice straw (UTRS) basal diets, and legume soilage supplement utilized in this study is presented in Table 1.0. The values reflected for DM, CP, NDF, and GE were obtained from the proximate analysis conducted at the Animal Nutrition Laboratory of the Department of Animal Science, Visayas State University, Philippines whereas the values reflected for lignin and tannin content of Napier and UTRS were obtained from the Feedipedia website (https://www.feedipedia.org), while that of the legume supplement was obtained from the study of Oden et al (2000).

As the feed offered was adjusted to the basal diets of the sheep with the lowest voluntary DMI and the rice straw was alkali treated, we expect that no significant difference will be observed in the intake and digestibility by the animals (Table 2). However, the tendency toward higher (P=0.0540) dry matter intake was observed for T2 and T4 treatments compared with T1 and T3. Intake relative to metabolic weight ($BW^{0.75}$) was high (P=0.0255) in T2 and T4. In terms of dry matter digestibility, the differences among treatments were not significant, although Napier grass treatments (T1 and T2) were numerically higher than the UTRS treatments (T3 and T4). Fiber intake (NDFi kg/day) (P=0.0046) was significantly higher in T1 and T3 whereas fiber digestibility (NDFD %) was similar (P=0.4357) among treatments. Numerically, the peak ratio (%) of methane to carbon dioxide (CH_4/CO_2) was relatively lower (P=0.5782) in treatments with legume supplements (T2 and T4) than with a pure basal diet alone (T1 and T3).

Table 2. Intake (kg), digestibility (%), and methane production (%) in sheep fed with two types of the basal diet with or without legume forage supplement

Parameters	Dietary Treatments				SEM	*p*-value	
	T1	T2	T3	T4		Trt	F1xF2
DMI (kg day^{-1})	0.227	0.265	0.249	0.263	0.01	0.0540	0.2361
DMI ($BW^{0.75}$)	0.026^{b}	0.037^{a}	0.027^{ab}	0.033^{ab}	0.002	0.0255	0.3087
DMD (%)	70.34	67.18	60.35	62.57	5.74	0.6192	0.6478
NDFi (kg day^{-1})	0.23^{a}	0.17^{b}	0.23^{a}	0.19^{b}	0.01	0.0046	0.1225
NDFD (%)	73.35	61.24	71.08	63.57	5.89	0.4357	0.7033
CH4/CO2	50.47	27.87	44.28	22.92	15.83	0.5782	0.9694
CH4/CO2 (%/$BW^{0.75}$)	6.70	3.95	4.84	2.84	2.27	0.6790	0.8705

[a,b,c] Means in the same row with different superscripts are significantly different (p<0.05)

T1=napier grass soilage; T2=napier grass soilage + legume; T3=urea-treated rice straw (UTRS); T4=urea-treated rice straw (UTRS) + legume; Trt=treatment: F1xF2= interaction effect between factor 1 and 2; DMI=dry matter intake expressed in kg day^{-1}; DMI ($BW^{0.75}$)=dry matter intake in relation to metabolic body weight; DMD=dry matter digestibility; NDFi=neutral detergent fiber intake; NDFD = neutral detergent fiber digestibility; SEM=Standard Error of the Mean; CH4/CO2=methane to carbon dioxide ratio; CH4/CO2 (% /$BW^{0.75}$)=methane to carbon dioxide ratio in relation to metabolic body weight

The rate and degree of fiber digestion influence ruminant fodder intake. Legumes often have a faster passage rate in the rumen due to their lower fiber content, quick fermentation, and particle disintegration (Martin et al 2016). Thus, adding highly fermentable OM-containing legume forage like *G. sepium* to a diet increases overall consumption. In this study, the faster rate of breakdown and shorter retention period in the rumen may be the cause of the observed increase in intake by the animals in that received dietary supplementation with legumes. In addition, treating rice straw with urea may significantly improve the fiber content's breakdown and increase feed intake while also improving nutrient digestibility and

passage rate (Gunun et al 2013). As a result, adding more legumes to animal diets will result in more degradable dry matter fractions, which will lead to increased intake. The age and level of lignification of the forage diets may be the cause of the Napier-based diets' numerically high dry matter digestibility. Theoretically, immature forages are higher in nutritional value and easier to digest than mature forages. According to Haryani et al (2018), harvesting Napier grass at 6 to 8 weeks of age will result in a decrease in CP and ME, as well as an increase in DM and CF than cutting at 4 weeks of age. The percentage of nutrients in Napier grass decreases with age, Mohamad et al (2022) recommend that the optimal time to harvest is 45 days. The Napier grass that was utilized in this study was cut at 45 to 60 days, which is in line with the time frame suggested to maximize dry matter yield and nutritional value. Urea treatment leads to swelling of the hemicelluloses-lignin complex in rice straw, resulting in an increased surface area available for attack by rumen microorganisms, thereby increasing the rate of breakdown and passage rate of rice straw through the digestive tract (Gunun et al 2013). In this study, the more lignified characteristics of urea-treated rice straw resulted in lesser digestibility than Napier-based diets. Comparing urea-treated rice straw supplementation or not, with legume (T3 vs T4) in this study, showed slightly increased digestibility, probably due to the lesser cell wall attribute of the legume. Compared to legumes, grasses have a high NDF concentration and typically lower intake potential, especially since their leaves are consumed more readily than stems. Grasses require more chewing because of their high cell wall concentration and do not fracture into small particles during chewing as readily as legumes (Buxton et al 1995). The low NDF intake observed with T2 and T4 was perhaps the result of its low concentration in the supplement which agrees with other reports (Ramirez-Aviles et al 1998, Orden et al 2000, Phelan et al 2015).

Rumen pH was similar (P=0.7041) across all dietary treatments (Table 3). The molar concentration of volatile fatty acid (VFA mmol/100mol) is significantly high (P=0.0303) in T1, which was found comparable with T2 and T3, while T4 has the lowest. There was a tendency (P<0.10) to increase the molar proportion of acetate (mmol/100mol) in T1 and T3, while the propionate concentration was significantly high (P<0.0001) in T2 and T4, respectively. A comparable high proportion of n-butyric acid (P=0.0005), (A/P) ratio and (A+B)/P (P<0.0001) were observed from T1 and T3, while T2 and T4 have the lowest molar proportion.

Table 3. Molar proportion and concentration (mmol L^{-1}) of volatile fatty acid in the rumen of sheep fed with two types of the basal diet with or without legume forage supplement

Parameters	Dietary Treatments				SEM	*p*-value	
	T1	T2	T3	T4		Trt	F1xF2
Rumen pH	6.50	6.68	6.51	6.49	0.13	0.7041	0.4514
Total VFA (mmol L^{-1})	21.65^{a}	18.95^{ab}	16.93^{ab}	14.53^{b}	1.48	0.0303	0.9208
Acetic acid (mmol 100mol^{-1})	56.52	54.40	55.23	54.51	0.67	0.1062	0.0389
Propionic acid (mmol 100mol^{-1})	23.17^{c}	28.56^{a}	23.50^{c}	26.23^{b}	0.51	<0.0001	0.0113
n-butyric acid (mmol 100 mol^{-1})	14.03^{ab}	12.25^{c}	14.60^{a}	12.43^{bc}	0.45	0.0005	0.6638
A/P	2.46^{a}	1.93^{c}	2.33^{ab}	2.12^{bc}	0.06	<0.0001	0.0070
(A+B)/P	3.07^{a}	2.37^{b}	2.96^{a}	2.60^{b}	0.01	<0.0001	0.0122

^{a,b,c} Means in the same row with different superscripts are significantly different (p<0.05)
T1=napier grass soilage; T2=napier grass soilage + legume; T3=urea-treated rice straw (UTRS); T4=urea-treated rice straw (UTRS) + legume; Trt=treatment; F1xF2=interaction effect between factor 1 and 2; VFA=volatile fatty acid; A/P=acetate to propionate ratio; (A+B)/P=(acetate + butyrate) to propionate ratio; SEM=standard error of the mean

Ruminant methane production is a by-product of the natural fermentation of feed by microorganisms in the rumen and, to a lesser extent, in the lower digestive tract. Methanogens, or methane-producing archaea, are essential for this process because they use hydrogen to convert CO_2 or formate to methane instead of allowing it to build up in the rumen. The rumen's bacterial, protozoal, and fungal populations ferment lipids, proteins, and carbohydrates to create the main volatile fatty acids such as acetate, propionate, and butyrate. The production of acetate and butyrate releases hydrogen, whereas the production of propionate acts as a net hydrogen sink. Because less hydrogen is generated when propionate is produced in the rumen, diets that do so frequently produce less methane. According to numerous reviews (Martin et al 2016, Beauchemin et al 2008, Mueller-Harvey et al 2019), the ability of legumes to inhibit the production of methane in ruminants is often explained by the presence of condensed tannins (CT), lower fiber content, higher DMI, and a faster rate of passage from the rumen, all of which were present in this study. Condensed tannins can reduce ruminants' methane emissions through several mechanisms: (1) directly suppressing the rumen-based methanogenic archaea's ability to produce methane during enteric fermentation. Tannins can disrupt these methanogens' cell membranes and metabolic processes, reducing their population and methane production; (2) in the rumen, where hydrogen gas (H_2) is produced as a byproduct of microbial fermentation and used by methanogens to produce methane, tannins can act as a hydrogen sink. Condensed tannins can form tannin-protein complexes in the rumen when they bind to food components or proteins. These complexes can act as hydrogen sinks by efficiently trapping hydrogen and preventing methanogens from using it to make methane. This lowers methane output without affecting rumen fermentation; (3) condensed tannins can also inhibit methane gas production by altering the rumen microbial populations. While they may inhibit methanogenic archaea, they can promote the growth of other bacteria that are less efficient at producing methane or that utilize alternative pathways for hydrogen disposal; and (4) in the rumen, condensed tannins reduce the digestibility of fibrous materials, such as cellulose and hemicellulose, which can lead to lower overall rumen fermentation (Aboagye et al 2019, Cardoso-Gutierez et al 2021).

Reduced substrate fermentation that results in lower CH_4 production is often linked to the low total VFA concentrations in the rumen (Knapp et al 2014), which were seen in animals supplemented with *G. sepium* (T2 and T4). The diet's rumen fermentation pattern controls the amount of hydrogen produced, which is then converted to CH_4. While high acetate production and, to some extent, butyrate, is related to high CH_4 generation, high propionate is associated with reduced hydrogen release and low CH_4 production (Hegarty et al 2007, McCauley et al 2020). Supplementing the diet of ruminant animals with legumes can increase propionate production in the rumen due to several factors: (1) legumes are high in protein compared to many other forage sources. When ruminants consume legumes, they provide a source of high-quality protein for the rumen microbes. This increased protein availability can stimulate the growth and activity of certain rumen bacteria that are efficient at producing propionate; (2) legumes are more easily digested than other forages, which implies they may be broken down in the rumen more easily. This enhanced digestibility leads to increased microbial fermentation in the rumen, including propionate synthesis; (3) legumes are also a rich source of nitrogen for rumen microorganisms. Nitrogen is required for microbial

development and activity in the rumen. When microorganisms have a sufficient nitrogen source, they may efficiently digest carbohydrates, resulting in increased propionate synthesis; (4) when compared to other forages, legumes often contain more soluble carbohydrates, such as sugars and starches. Rumen bacteria may rapidly ferment these soluble carbohydrates, producing propionate as a main end product; (5) finally, as compared to grasses and other forages, legumes have lower fiber content and a broader C: N (carbon-to-nitrogen) ratio. A lower C: N ratio promotes the development of bacteria that create propionate during fermentation, which is beneficial for propionate production.

This study found a significant shift in the production of propionate, with less acetate and butyrate produced for T2 and T4. This shift may be related to the condensed tannins in the legume *G. sepium*, which have an inhibitory effect on cellulolytic bacteria by inactivating their extracellular enzymes, inhibiting the production of acetate and H_2, and reducing their digestive activity (Martin et al 2016). Furthermore, legumes' increased propionate concentration, digestibility, nitrogen supply, carbohydrate composition, and reduced fiber content support the proliferation and activity of propionate-producing bacteria in the rumen. As a result, feeding legumes to ruminants can enhance propionate synthesis, which is an essential volatile fatty acid used by the animal as an energy source and in a variety of metabolic activities.

CONCLUSION

This study confirmed the potential of *G. sepium* as a methane-mitigating supplement to basal diets such as Napier and urea-treated rice straw, as feed for ruminants. Generally, based on the result, the addition of *G. sepium* promotes a high passage rate that tends to increase dry matter intake of both Napier and UTRS and reduces the availability of substrate in the rumen which leads to lower total VFA production. The presence of the secondary plant compound, tannin, in the *G. sepium* significantly increased rumen propionate, which serves as a sink for excess hydrogen while acetate and butyrate production that releases hydrogen, available for methanogenesis, was suppressed. This was reflected numerically by a lower methane to carbon dioxide ratio of almost 50%. Feeding 6 to 8 weeks old Napier grass alone optimizes its nutritive value while urea-treatment of rice straw improves its palatability. The feeding potential of these basal diets further improves when mixed with legumes, and reduces enteric methane gas emissions.

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