Article

In Vitro Screening of Plant Materials to Reduce Ruminal Protozoal Population and Mitigate Ammonia and Methane Emissions

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Abstract: Alternative feed sources can be utilized to reduce enteric methane (CH₄) emissions, a major greenhouse gas that contributes to global warming. This study aimed to evaluate the potential use of tropical plants to improve digestibility, reduce protozoal populations, improve rumen fermentation, and minimize methane emissions from ruminants. The plants considered herein grow in tropical climates, are easily accessible in large quantities, and are directly related to human food production. Nine plants that grow naturally in tropical climates were assessed. Plant supplementation substantially enhanced accumulative gas production at 24 h ($p < 0.05$). The apparent organic matter digestibility (AOMDvt) of the diet was not affected by five of the nine plants. With the addition of the plant material, ammonia nitrogen concentrations were reduced by up to 47% and methane concentrations were reduced by 54%. Five of the nine plant materials reduced methane production in terms of CH₄/dry matter and CH₄/digestibility of the organic matter by 15–35% and 8–24%, respectively. In conclusion, supplementation with plants with high tannin contents was shown to be a viable strategy for improving rumen fermentation, reducing protozoal populations, and limiting methane emissions. In this regard, the leaves of *Piper sarmentosum*, *Acmella oleracea*, *Careya arborea*, and *Anacardium occidentale* were especially promising.

Keywords: phytochemical; protozoa; ruminal fermentation; tannins; methanogenesis; ammonia

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1. Introduction

Livestock production has long been important in agricultural farming in developing countries. It is essential for the production of meat, dairy, and agricultural dung, and has a significant effect on regional stability and improving livelihoods [1]. The animal production industry is thought to contribute up to 16.5% of global greenhouse gas (GHG) emissions, which have become a major concern in recent decades [2,3]. Enteric fermentation and feed production activities, which account for almost 45% of the sector's overall emissions, are the main source of GHG emissions in ruminant agriculture [4]. Methane (CH₄) is the second most important GHG emitted by human activities [5]. Enteric CH₄ is produced primarily in the rumen by methanogenic archaea, which convert the hydrogen (H₂) and carbon dioxide (CO₂) produced by a diverse community of microorganisms through fermentation [6]. Furthermore, methane production provides for approximately 5–7% of the feed gross energy, or nearly 16–26 g/kg of feed consumed [7], and it is often regarded as a source of energy loss for the animal. As a result, the use of plant secondary metabolites is one of the primary options being investigated for reducing enteric CH₄ in this sector [8–10]. Secondary metabolites (tannins, saponins, etc.), which are found in many plant species, were found to have the capacity to modify the rumen methanogenic bacteria population. The use of such feed products may be an effective way to decrease methane

emissions by improving the overall rumen ecosystem and, as a result, ruminant animal productivity by properly using secondary compounds in tropical plants [11]. Furthermore, tropical regions across the world have a diverse variety of forage resources with favorable chemical compositions that could be used as feed for livestock production systems.

Research into nutrient-rich local resources is critical for improving the cost and efficiency of tropical livestock systems while also reducing their environmental effects. The tropical environment has many natural resources, including fodder trees, shrubs, and herbaceous plants, which have the potential to reduce CH₄ emissions from cattle while also recovering deteriorated areas [11,12]. Tropical plants have many uses, including as food flavoring agents, in traditional medicine, and for synthetic insecticides. Certain plant species have been shown to include alkaloids, xanthophylls, polyphenols, lignans, organosulfide, flavonoids, tannin, and saponin. Moreover, the negative effects of flavonoid-rich plants on CH₄ emissions and methanogenic bacteria were determined in vitro [13] and in vivo [14,15]. However, phytochemicals, such as secondary compounds and essential oils, have consistently demonstrated consistent results, varying from a significant reduction to a modest increase in enteric CH₄ emissions [16]. The aim of this research was to investigate the in vitro fermentation, protozoal population, and enteric methane mitigation capability of nine plants with high polyphenol or tanniniferous contents.

2. Materials and Methods

The study was conducted at the Animal Science Research Unit, the Department of Agriculture and Resources, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Thailand. Animal procedures were approved by the Animal Ethics Committee of Kasetsart University (record no. ACKU64-CSC-004-19/07/2021), based on the Ethic of Animal Experimentation of National Research Council of Thailand.

2.1. Plant Materials Preparation

In the present study, plant samples (rhizome, leaves, and tender stems) were hand harvested in Sakon Nakhon Province, Thailand, during the seasons indicated (Table 1). The following nine plant materials were tested: the rhizome of *Zingiber officinale* (ginger), *Alpinia galanga* (galanga), *Zingiber cassumunar* (cassumunar ginger), and *Curcuma longa* (turmeric); the stem and leaf part of *Cymbopogon citratus* (lemon grass), *Piper sarmentosum* (wild pepper), and *Acmella oleracea* (toothache plant); and the leaf of *Careya arborea* (Ceylon oak) and *Anacardium occidentale* (cashew nut). Farmers' plots from three districts, i.e., Sakon Nakhon Province, Maung Sakon Nakhon, Phonnakeaw, and Phuphan, were randomly selected to harvest all plant material. The plant materials were dried at 45 °C in a hot air oven dryer to a consistent weight before being mashed through a 0.1 cm mesh (Polymix PX-MFC 90D, Kinematica, Switzerland).

Table 1. The scientific names, plant parts, and harvesting times of the plant samples tested (*n* = 3).

Plant Species.	Plant Family	Common Name	Plant Part	Harvest Time
<i>Zingiber officinale</i> Roscoe	Zingiberaceae	Ginger	Rhizome	July
<i>Alpinia galanga</i> L.	Zingiberaceae	Galanga	Rhizome	September
<i>Cymbopogon citratus</i>	Poaceae	Lemon grass	Stem + Leaf	July
<i>Zingiber cassumunar</i> Roxb.	Zingiberaceae	Cassumunar ginger	Rhizome	September
<i>Curcuma longa</i> L.	Zingiberaceae	Turmeric	Rhizome	September
<i>Piper sarmentosum</i> Roxb.	Piperaceae	Wild pepper	Stem + Leaf	July
<i>Acmella oleracea</i> L.	Asteraceae	Toothache plant	Stem + Leaf	May
<i>Careya arborea</i> Roxb.	Lecythidaceae	Wild guava, Ceylon oak	Leaf	May
<i>Anacardium occidentale</i> L.	Anacardiaceae	Cashew nut	Leaf	May

2.2. Chemical Composition Analysis

Plant materials, roughage, and concentrate samples were chemically analyzed for dry matter (DM), organic matter (OM), ash, and crude protein (CP), according to the method of AOAC [17]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) in substrates were determined according to Van Soest et al. [18], adapted to an Ankom Fiber Analyzer A2000 (Ankom Technology Corp., Macedon, NY, USA). Ether extract content was determined using the Soxhlet method. Total phenols (TP) were estimated using the Folin–Ciocalteu reagent method [19]. According to spectrophotometer measurements, the condensed tannin (CT) fraction was determined using the HCl-butanol method described by Terrill et al. [20].

2.3. Rumen Collection

In this experiment, three crossbred steers weighing 380 ± 28 kg each were used as rumen fluid donors. Total mix ration (TMR) diet (basal diet in Table 2) was offered to the animals at a daily intake of 2.5% of their body weight (BW). The cattle were provided access to mineral blocks and kept in separate cages with clean water. Rumen fluid was collected using stomach tube with a vacuum pump prior to feeding TMR in the morning (06.00 a.m.), filtered through cheesecloth into warmed flasks, and then transported to the laboratory. Before preparing a batch culture, ruminal fluid from the three cattle was mixed in equal proportions.

Table 2. Ingredients and chemical composition of the basal diet.

Items	Concentrate	Rice Straw
Ingredients (% DM)		
Cassava chips	58.5	
Rice bran	14.0	
Palm kernel meal	14.0	
Soy bean meal	6.0	
Mineral mixed *	1.0	
Sulfur	1.0	
Urea	1.5	
Salt	1.0	
Molasses	3.0	
Chemical composition		
Dry matter (%)	93.5	94.5
Organic matter (% DM)	92.7	93.1
Crude protein (% DM)	14.0	2.8
Neutral detergent fiber (% DM)	12.5	65.8
Acid detergent fiber (% DM)	8.9	43.3

* Mineral (each kg contain): Fe 45 g; Zn 30 g; Mn 60 g; Co 0.01 g; Cu 5 g; Se 0.04 g; I 0.01 g.

2.4. In Vitro Gas Production

The in vitro method described by Menke and Steingass [21] was used to estimate gas production. Two sets of modified 100 mL glass syringes were used for incubation: one for fluid sampling and the other for gas sampling. In this study, 15 mg of plant material were supplemented into 200 mg of basal diet (in DM basis). TMR (basal diet) was comprised of rice straw and concentrate in a 70:30 ratio (Table 2). Samples with plant supplementation were accurately weighed into 100 mL glass syringes fitted with plungers. The solution was then dissolved in a 1:2 ratio with a buffer according to Menke and Steingass' technique [21]. In the laboratory, the obtained solutions were mixed together in one beaker under a continuous stream of CO₂ and maintained in a water bath at 39 °C before being added to the syringes. Rumen liquid was filtered through four layers of gauze and mixed in a 1:2 ratio with a buffer solution according to Menke and Steingass' technique [21]. For each treatment, 30 mL of rumen fluid-buffer mixture was loaded into

syringes (six runs per plant material sample). Each run contained a duplicate of blanks and two syringes, with the basal diet as a negative control for a total of 64 incubations.

2.5. In Vitro Rumen Fermentation Characteristics

The syringes were put into a 39 °C incubator, and the accumulative gas volume was measured using the calibrated scale written on the syringes after 2, 4, 8, 12, 24, 36, and 48 h of incubation. The data for cumulative gas were fitted to Ørskov and McDonald's model [22]. A pH meter was used to measure the pH of the incubation solution immediately after collection (pH: HI 8424 microcomputer pH meter, Singapore). After 24 h of inoculation, the incubation liquid was collected and analyzed for ammonia nitrogen (NH₃-N) content [17], with only distillation and titration steps. The concentration of volatile fatty acid (VFA) was analyzed using high-performance liquid chromatography (Agilent 1200 series, Agilent Technologies Inc. Santa Clara, CA, USA) with a diode array detector (Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm)) using 0.1 M phosphate buffer as the mobile phase. A total of 150 µL of fermentation gas was collected from the incubation syringes and injected into a gas chromatograph using a gas-tight Hamilton syringe (Agilent Technologies Inc. 5977B Single Quadrupole, Bellefonte, PA, USA) with the following specifications: Carboxen 1000, 45/60, 2 m 1/8" (Supelco, Bellefonte, PA, USA) column with flame ionization detector. In vitro digestibility was determined after 24 h of incubation when the contents were filtered through pre-weighed Gooch crucibles and residual dry matter was measured after 48 h of oven drying at 60 °C. The residual sample from each was utilized for AOMDvt following Tilley and Terry's method [23]. Net energy for lactation (NE_L) was estimate using the equation of Menke and Steingass [21] as given below (Equation (1)).

$$NE_L \text{ (MJ/kg DM)} = -0.22 + 0.1062 \text{ GP} + 0.048 \text{ CP} + 0.1329 \text{ EE} \quad (1)$$

where GP denotes 24 h of net gas production (mL/200 mg), CP denotes crude protein (%), and EE denotes ether extract (%).

2.6. Bacterial and Protozoal Count

After 24 h of fermentation, a 1 mL sample of the incubation liquid was immediately added to 6 mL of 10% formaldehyde. The population of protozoa and bacteria were determined using the method of Galyean [24]. The incubation fluid was diluted with 10 times the amount of autoclaved distilled water and protozoa and bacteria were counted with a hemacytometer (Boeco, Hamburg, Germany) and under a light microscopic (150×) (Olympus BX51-DIC-B, Olympus Optical Co. Ltd., Tokyo, Japan).

2.7. Statistical Analysis

All data were obtained using the SAS general linear model procedure (SAS Institute Inc., Cary, NC, USA). Tukey's test was used to examine differences between treatment means, and differences between means with $p < 0.05$ were considered statistically significant. The statistical model and experimental design were as follows:

$$Y_{ij} = \mu + M_i + \varepsilon_{ij} \quad (2)$$

where Y_{ij} denotes the observation variable, μ denotes the overall mean, M_i denotes the influence of the plant material ($i = 1-9$), and ε_{ij} denotes the residual effect. The incubation run ($n = 6$) was categorized as a random effect ($n = 6$), whereas the plant material (including the basal diet alone) was classified as a fixed effect ($n = 10$).

3. Results

3.1. Chemical Analyses of Plant Materials

Table 3 shows the chemical analysis of the plant materials used in this study. The crude protein levels varied from 54.8 to 234.5 g/kg DM. The highest levels were detected in *Acmella oleracea* (234.5 g/kg DM) and the lowest in *Zingiber officinale* (76.9 g/kg DM).

EE was found in small quantities in *Anacardium occidentale* (11.9 g/kg DM), followed by *Cymbopogon citratus* (18.7 g/kg DM); in contrast, almost eight times as much EE was detected in *Curcuma longa* (about 80 g/kg DM). NDF and ADF levels were highest in *Cymbopogon citratus* (649.4 and 424.0 g/kg DM), followed by *Piper sarmentosum* (647.2 and 398.6 g/kg DM), and lowest in *Curcuma longa* rhizome (404.9 and 131.9 g/kg DM). Among all the plant materials, *Careya arborea* and *Anacardium occidentale* had the highest CT content (159.2 and 165.7 g/kg DM, respectively), whereas *Alpinia galangal* had the highest TP levels (385.4 g/kg DM).

Table 3. Chemical composition of the plant samples tested ($n = 3$).

Scientific Names	Chemical Composition (g/kg DM)						
	OM	CP	EE	NDF	ADF	CT	TP
<i>Zingiber officinale</i>	832.2	76.9	63.6	563.2	369.7	27.6	155.8
<i>Alpinia galanga</i>	831.5	81.8	68.1	625.0	368.1	63.9	385.4
<i>Cymbopogon citratus</i>	897.4	54.8	18.7	649.4	427.0	15.4	24.6
<i>Zingiber cassumunar</i>	957.0	136.2	58.8	423.8	184.7	14.0	236.4
<i>Curcuma longa</i>	952.5	104.7	78.8	404.9	131.9	29.4	289.6
<i>Piper sarmentosum</i>	940.1	188.5	63.9	647.2	398.6	89.3	91.2
<i>Acmella oleracea</i>	946.8	234.5	52.2	434.1	189.3	104.1	135.7
<i>Careya arborea</i>	966.0	150.3	59.8	514.1	309.3	159.2	201.5
<i>Anacardium occidentale</i>	928.3	97.5	11.9	458.4	391.5	165.7	194.3

OM: organic matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; CP: crude protein; CT: condense tannins; TP: total phenol.

3.2. In Vitro Gas Production Parameters

The gas production kinetic parameters and cumulative gas production parameters at 48 h of incubation are shown in Table 4. The quantity of gas produced from the rapidly soluble fractions (a), the amount of gas produced from the insoluble fraction (b), and the potential extent of gas produced (a + b) all varied considerably ($p < 0.05$). The insoluble fraction (c) gas production rate constants, on the other hand, exhibited no statistically significant influence when plant materials were added ($p > 0.05$). Adding *Zingiber cassumunar* and *Curcuma longa* to a 200 mg basal diet lowered the kinetic gas parameters. After 48 h of incubation, a similar pattern was found in cumulative gas generation, which was statistically enhanced when tree leaf foliage was added ($p < 0.05$); however, rhizomes in the substrate lowered the cumulative gas production.

Table 4. The effect of plant supplementation on the in vitro gas kinetics and cumulative gas production at 48 h after incubation.

Treatments	Gas Production Kinetic Parameters				Cumulative Gas (mL/48 h)
	a	b	c	a + b	
Basal diet	2.07 ^{ab}	102.60 ^f	0.062	104.53 ^d	99.80 ^e
<i>Zingiber officinale</i>	3.05 ^{bc}	78.44 ^{bc}	0.060	81.49 ^b	80.15 ^b
<i>Alpinia galanga</i>	1.36 ^a	73.96 ^{ab}	0.052	75.42 ^a	72.90 ^a
<i>Cymbopogon citratus</i>	2.02 ^{ab}	84.22 ^c	0.050	86.42 ^b	84.95 ^c
<i>Zingiber cassumunar</i>	1.35 ^a	72.04 ^a	0.034	73.39 ^a	70.40 ^a
<i>Curcuma longa</i>	1.74 ^a	72.86 ^a	0.030	73.59 ^a	70.70 ^a
<i>Piper sarmentosum</i>	1.91 ^a	80.74 ^c	0.043	82.65 ^b	81.90 ^b
<i>Acmella oleracea</i>	3.41 ^c	96.42 ^e	0.048	99.83 ^d	98.40 ^e
<i>Careya arborea</i>	2.18 ^{ab}	80.69 ^c	0.060	82.79 ^b	80.50 ^b
<i>Anacardium occidentale</i>	2.32 ^{ab}	90.55 ^d	0.054	92.87 ^c	91.55 ^d
SEM	0.325	1.706	0.011	1.723	0.956

^{a–f} Significant difference with $p < 0.05$; SEM: standard error of the mean.

3.3. In Vitro Rumen Fermentation, Bacterial, and Protozoal Population Characteristics

After a 24-h incubation period, the fermentation fluid had a pH range of 6.6–6.9. Table 5 shows that all plant materials reduced $\text{NH}_3\text{-N}$ concentrations by 47% as compared to the basal diet, especially when *Anacardium occidentale* was supplemented ($p < 0.05$). The bacterial count was affected by certain plant materials ($p < 0.05$), e.g., a high count was observed with *Cymbopogon citratus* meal and a low count was observed with *Curcuma longa*. Protozoal counts were significantly decreased as a result of all plant materials supplements when compared to the basal diet; however, *Zingiber officinale* did not have an effect ($p < 0.05$). The total volatile fatty acid (TVFA) concentration decreased after supplementation with all plant materials ($p < 0.05$). The propionate (C3) proportion in plant material additives was significantly altered, particularly in *Cymbopogon citratus*, *Acmella oleracea*, and *Careya arborea*, but acetate (C2) and butyrate (C4) were not changed as compared to the basal diet. *Curcuma longa* had the greatest acetate to propionate (C2:C3) ratio (3.78), while *Careya arborea* had the lowest at 3.39 ($p < 0.05$).

Table 5. The effects of basal diet as a control substrate and plant supplementation on ruminal fermentation parameters.

Plant Species	Ammonia (mg/dL)	Total Bacteria (10^9 /mL)	Total Protozoa (10^4 /mL)	SCFA (mmol/dL) and the Molar Proportions (mmol/100 mol)				
				TVFA	C2	C3	C4	C2/C3
Basal diet	13.98 ^c	5.76 ^c	5.73 ^c	94.19 ^c	69.61	18.24 ^a	11.85	3.75 ^d
<i>Zingiber officinale</i>	9.85 ^{ab}	4.48 ^{bc}	4.96 ^{bc}	87.64 ^{ab}	69.75	18.98 ^{abc}	11.27	3.67 ^{bcd}
<i>Alpinia galanga</i>	8.94 ^{ab}	4.82 ^{bc}	4.32 ^b	85.60 ^a	69.80	18.69 ^{abc}	11.51	3.73 ^{cd}
<i>Cymbopogon citratus</i>	9.45 ^{ab}	5.05 ^{bc}	2.25 ^a	88.78 ^{ab}	69.20	19.65 ^{bc}	11.15	3.52 ^{ab}
<i>Zingiber cassumunar</i>	9.52 ^{ab}	3.14 ^b	2.64 ^a	86.28 ^{ab}	69.96	18.42 ^{ab}	11.62	3.80 ^d
<i>Curcuma longa</i>	10.70 ^b	1.26 ^a	2.04 ^a	85.15 ^a	69.56	18.40 ^{ab}	12.04	3.78 ^d
<i>Piper sarmentosum</i>	9.38 ^{ab}	4.46 ^{bc}	2.88 ^a	89.30 ^{bc}	68.93	19.54 ^{abc}	11.53	3.53 ^{abc}
<i>Acmella oleracea</i>	9.78 ^{ab}	5.00 ^{bc}	2.91 ^a	85.48 ^a	68.31	19.72 ^{bc}	10.97	3.46 ^a
<i>Careya arborea</i>	8.59 ^{ab}	3.75 ^b	2.72 ^a	90.5 ^{bc}	68.96	20.29 ^c	10.75	3.39 ^a
<i>Anacardium occidentale</i>	7.39 ^a	4.25 ^{bc}	2.33 ^a	84.58 ^a	69.18	18.62 ^{ab}	12.20	3.72 ^{bcd}
SEM	0.741	0.582	0.439	1.395	0.634	0.487	0.351	0.063

^{a–d} Significant difference $p < 0.05$; SCFA: short-chain fatty acids; TVFA: total volatile fatty acids; C2: acetate; C3: propionate; C4: butyrate; C2/C3: acetate/propionate ratio; SEM: standard error of the mean.

3.4. In Vitro Apparent OM Digestibility, Methane Production, and Net Energy for Lactation

In comparison to the basal diet, supplementation with plant materials had no effect on total gas production during the 24-h incubation period. Supplementation with *Curcuma longa*, *Careya arborea*, and *Anacardium occidentale*, on the other hand, considerably reduced the total gas volume ($p < 0.05$). In vitro apparent organic matter digestibility (AOMDvt) and the amount of organic matter (OM) digested in 24 h both decreased with the addition of *Curcuma longa*, *Acmella oleracea*, *Careya arborea*, and *Anacardium occidentale* ($p < 0.05$) (Table 6). In comparison to the control, all plant material supplements reduced methane (CH_4) production per digested unit of OM (dOM) ($p < 0.05$). Methane production per unit of ingested OM and total CH_4 production in mL/24 h were both similar. *Zingiber officinale* had the smallest effect; whereas *Acmella oleracea* and *Careya arborea* had the greatest CH_4 -mitigating effect. The CH_4 to TVFA ratio was dramatically lowered as a result of all plant materials, with the addition of *Careya arborea* yielding the lowest value ($p < 0.05$) (Table 4). Supplementing four plant materials to a level ranging from 0.47 (*Careya arborea*) to 0.79 (*Anacardium occidentale*) MJ/kg DM reduced the NE_L content of the basal diet (Table 6).

Table 6. The effects of plant supplementation with basal diet on gas production, in vitro apparent organic matter digestibility, methane production, and NE_L content in DM.

Plant Species	Total Gas (mL/24 h)	AOMDvt (%)	dOM (mg/24 h)	CH ₄ /OM (mL/g)	CH ₄ (mL/24 h)	CH ₄ /TVFA (mmol/mol)	NE _L (MJ/kg DM)
Basal diet	69.24 ^{cd}	67.62 ^c	135.24 ^c	57.01 ^f	7.71 ^e	132.19 ^e	5.16 ^d
<i>Zingiber officinale</i>	75.20 ^d	67.28 ^c	134.56 ^c	50.16 ^e	6.75 ^d	116.09 ^d	4.68 ^{abc}
<i>Alpinia galanga</i>	70.78 ^{cd}	66.55 ^c	133.10 ^{cd}	38.47 ^c	5.12 ^c	110.45 ^c	5.21 ^d
<i>Cymbopogon citratus</i>	66.80 ^c	66.80 ^c	133.60 ^{cd}	39.75 ^c	5.31 ^c	108.88 ^c	4.50 ^{ab}
<i>Zingiber cassumunar</i>	65.88 ^c	65.89 ^{bc}	131.78 ^{bcd}	32.79 ^b	4.32 ^b	100.04 ^a	5.06 ^{cd}
<i>Curcuma longa</i>	58.09 ^{ab}	59.96 ^a	119.92 ^a	43.95 ^d	5.27 ^c	110.96 ^c	4.96 ^{cd}
<i>Piper sarmentosum</i>	65.95 ^c	64.50 ^b	129.01 ^{bc}	33.64 ^b	4.34 ^b	104.57 ^b	4.92 ^{bcd}
<i>Acmella oleracea</i>	63.80 ^{bc}	63.84 ^b	127.68 ^b	28.52 ^a	3.64 ^a	101.65 ^{ab}	5.00 ^{cd}
<i>Careya arborea</i>	53.67 ^a	59.85 ^a	119.70 ^a	29.49 ^a	3.53 ^a	99.29 ^a	4.69 ^{abc}
<i>Anacardium occidentale</i>	53.92 ^a	58.96 ^a	117.92 ^a	32.74 ^b	3.86 ^{ab}	104.71 ^b	4.37 ^a
SEM	0.824	0.761	1.523	0.695	0.194	1.145	0.135

^{a–f} Significant difference $p < 0.05$; CH₄: methane; AOMDvt: apparent organic matter digestibility in vitro [25]; dOM: digestibility of organic matter; TVFA: total volatile fatty acids; SEM: standard error of the mean.

4. Discussion

4.1. Chemical Analyses of Plant Materials

In the concentrate diet, cassava chips were utilized as an energy source, providing 58.5% of the DM, and urea was used as a fermentable nitrogen source (1.5% DM). A substrate containing rice straw (CP at 2.8% DM) was used as a roughage source, and the concentrate diet comprised 14.0% CP. Carious factors, including species, season, and geographical zone, can alter the composition of woody plants, which vary considerably in terms of nutritional and CT content, and methanogenesis capacity [26]. Tannin and total phenolic component concentrations in plant material may impact the protozoal population and rumen fermentation (Table 5). Research into natural resources with high nutritional values is important for enhancing the profitability and productivity of livestock systems in tropical regions, and reducing their environmental impact [8–10,12–14]. Several mitigation strategies are associated with an increase in animal production efficiency based on various nutritional and environmental improvements [27]. Furthermore, as a result of the obvious strong interest in CT biological activity in the bovine habitat, it is important to mention that not all forms of CT are beneficial for ruminant nutrition. *Anacardium occidentale* had the greatest CT content (165.7 g/kg DM) in our study, followed by *Careya arborea* (159.2 g/kg DM) and *Acmella oleracea* (104.1 g/kg DM). Despite having a higher CT content than the other plants investigated, *Anacardium occidentale* did not produce the best biological effect. Various plants, particularly the ginger plant or the Zingiberaceae family, are widely used as a spice and flavoring agent in foods, but can also be added to animal feed to improve productivity, e.g., as a flavoring, to increase digestion and performance, for stress management, to enhance animal welfare, for example, in the form of odor stabilizers, to improve the local environment, and as antibacterial agents, such as is the case for certain essential oils that can replace in-feed antibiotics [27,28].

4.2. In Vitro Gas Production Parameters

It has been demonstrated that the availability of nutrients in plant material stimulates kinetic gas production. This was supposed to be attributed in part to the availability of plant material in the mixture, which supplied soluble carbohydrates (molasses) and protein (urea). This was thought to be when the basal diet included plant material, which provided soluble carbohydrates (molasses) and protein (urea). In vitro gas production has been shown to be a more efficient method of evaluating tannin effects than in sacco and is an indirect measure of substrate degradation [29,30]. The effects of tannins from diverse sources on ruminal gas production and ammonia quantities have been studied [31]. There was a consistent increase in gas production over a 48-h period; moreover, there were

significant differences in the total gas volume between plant supplementations (Table 4). Supplements with low tannin levels provided the most net gas, whereas additions with higher tannin levels produced the lowest. Getachew et al. [32] and Terranova et al. [33] demonstrated strong connections between CT and gas production, and their findings are consistent with ours. According to Cherdthong et al. [13], a positive correlation between gas production and high-CT pellet supplementation could be due to microbial activity stimulation caused by increased adverse microbiological conditions. The lower gas output for tannin treatments as compared to the control indicated that high tannin levels in plant material supplements limited ruminal carbohydrate fermentation [34]. This finding was enhanced in part by the fact that high-CT plant materials supplementation resulted in significantly lower VFA concentrations. However, high CT and TP contents in plant materials in the substrate may have a negative effect on rumen microbial activity, decreasing gas kinetics and production [33,35].

4.3. In Vitro Rumen Fermentation, Bacteria, and Protozoal Population Characteristics

The plant supplementation had an in vitro ruminal pH of 6.6–6.9, which was within the typical range of ruminal pH of 5.5–7.0. In contrast to the control, increased tannin in the plant materials decreased TVFA concentrations, which is in accordance with the findings of Wanapat et al. [14] and Min et al. [36]. Reduced $\text{NH}_3\text{-N}$ concentrations indicate increased nitrogen utilization by rumen bacteria for MCP production. The first case was substantiated by the lower molar proportions of branch chain volatile fatty acids, i.e., end products of the deamination of feed amino acids, in plant supplementation as compared to the control; the second case was supported by the higher MCP concentrations in the supplementation as compared to the control. Moreover, Aderinboye and Olanipekun [37] found that turmeric inclusion above 5 mg/g DM of substrate can modify the rumen by causing a reduction in fermentation end products, and a reduction in ammonia production at 15 mg/g, which significantly reduced microbial biomass, has implications for lowering microbial protein synthesis. In this study, both occurrences were confirmed in the plant materials with high tannin contents. Similarly, decreasing ruminal protozoal populations were related to CT and TP contents in plant material supplementations, particularly the addition of *Curcuma longa*, which reduced protozoa by 64% when compared to a basal diet. Methane production was negligible as compared to protozoal number. The protozoal population was reduced (Table 5) and methane production was decreased by supplementing with plant materials with high CT and TP concentrations in the fermentation substrate (Table 6). This finding was consistent with those of Sarnataro et al. [38], who found that adding *Stevia rebaudiana* Bertoni extract and chestnut wood tannin to the rumen reduced the in vitro protozoal population while having no effect on the fermentation parameters; whereas Jayanegara et al. [39] found dietary tannins had no effect on protozoal numbers in an in vitro study. Newbold et al. [40] recommended complete rumen protozoal elimination as a means to increase microbial protein supply by 30% while reducing methane production by up to 11%. However, Dai and Faciola [41] recommended partial rumen protozoal reduction strategies, and tannin supplementation, to reduce methane production. Plant material supplementation resulted in reduced TVFA concentrations, supporting the findings of Min et al. [36] concerning lower TVFA concentrations as compared to the control. This could be the result of decreased DM and NDF degradation as was shown by Chen et al. [34]. In the present study, nine of the plant additives resulted in a higher proportion of propionate, while practically all of the plant additives had no effect on the proportions of acetate and butyrate. As a result, these nine plant components reduced the acetate-to-propionate ratio. This study found an increase in propionate, which is similar to the findings of Cieslak et al. [42] who focused on *Vaccinium vitis idaea* extract supplementation, Wang et al. [43] who focused on *Atractylodes* rhizome and Amur cork tree supplementation, and Chen et al. [34] who focused on tannins. Propionate synthesis and methanogenesis are two competing processes for hydrogen metabolism in the rumen. As a result, processes that increase propionate may result in decreased methanogenesis, as was demonstrated

by the findings of this study. Furthermore, Zhou et al. [44] reported that feeding *Piper sarmentosum* extract at 1200 mg/kg decreased the ratio of acetate to propionate and reduced the population of protozoa, fungi, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* in goats.

4.4. In Vitro Apparent OM Digestibility, Methane Production, and Net Energy for Lactation

The inclusion of plant materials had no effect on total gas production throughout the 24-h incubation period in the current study. Furthermore, AOMDvt was comparable to that of the basal diet for several of the plant supplements. AOMDvt and total gas production were likewise lowest in plant materials with the lowest TVFA content. This may be related to the total bacteria and TVFA formation, as shown in Table 5. However, Akanmu et al. [45] observed that using plant extracts increased AOMDvt, while Cieslak et al. [42] showed that utilizing *Vaccinium vitis idaea* extract decreased methane without interfering with feed digestibility. According to Cielak et al. [46], plant secondary metabolites reduce nutrient digestibility, but only at higher levels of *Sanguisorba officinalis* supplementation. Nevertheless, Akanmu et al. [47] observed that plant extracts had a significant defaunating impact in the rumen, increasing the richness of bacteria, ruminal cellulolytic, and fungus populations. The reduction in ruminal fibrolytic bacterial populations caused tannins and saponins to have an inhibitory effect on nutrient digestion [27,34,44]. The addition of plant materials rich in CT and TP to ruminant feed is a promising strategy for reducing CH₄ emissions. Supplementation with *Careya arborea* reduced the CH₄-mitigating influence of the plant materials used in this study by up to 54%, which was much more pronounced than Cherdthong et al. [13,15] reported. This may be due to the difference in the basal diet. Similarly, supplementing the basal diet with these plant materials resulted in low NE_L, CH₄/OM, and CH₄/TVFA levels. Regardless of the fact that methanogenic archaea use H₂ and CO₂ as substrates to produce ruminal CH₄, it is generally known that tannins can reduce ruminal CH₄ production in two ways: (1) by inhibiting the activity of several rumen microbes affected in CH₄ production; and (2) by reducing carbohydrate digestion by forming stable complexes with carbohydrates [48–50]. Chen et al. [34] found that plant extracts containing both HT and CT had a stronger capacity to decrease methanogenesis than those containing only HT. The current study's reduction in CH₄ revealed that plants known to contain secondary metabolites are capable of inhibiting methanogenesis, and this was most probably due to a reduction in fiber degradation [50,51].

5. Conclusions

Methane formation per unit of digestible OM was reduced by five out of the nine plant materials as compared with the basal diet. Moreover, all materials decreased ammonia formation. The majority of the plant supplements tested had no adverse effect on in vitro digestibility. Plant materials, such as the leaves of *Piper sarmentosum*, *Acmella oleracea*, *Careya arborea*, and *Anacardium occidentale*, should be used as primary substrates in future in vitro investigations. Furthermore, future in vivo experiments should focus on the palatability of plant materials, confirm their mitigating effects, and evaluate their effect on production.

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