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Mitragyna speciosa Korth Leaves Supplementation on Feed Utilization, Rumen Fermentation Efficiency, Microbial Population, and Methane Production In Vitro

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Abstract: The objective of the research was to evaluate the different levels of *Mitragyna speciosa* Korth leaves powder (MSLP) added to rations with 60:40 or 40:60 roughage to a concentrate (R:C ratio) on in vitro nutrient digestibility, rumen fermentation characteristics, microbial population, and methane (CH_4) production. The treatments were arranged according to a 2 × 8 factorial arrangement in a completely randomized design. The two factors contain the R:C ratio (60:40 and 40:60) and the levels of MSLP addition (0, 1, 2, 3, 4, 5, 6, and 7% of the total substrate). There was no interaction between the R:C ratio and MSLP supplementation on gas production kinetics, ammonia nitrogen (NH_3-N), and microbial populations. The gas production rate constant for the insoluble fraction (c) was increased by the R:C ratio at (40:60), whilst there was no difference obtained among treatments for cumulative gas production, whilst the gas production rate constant for the insoluble fraction (c) was increased by the R:C ratio at 40:60. The concentration of NH_3-N was influenced by the R:C ratio and MSLP addition both at 4 and 8 h after incubation. In vitro dry matter degradability (IVDMD) and organic matter degradability (IVOMD) were significantly improved by the R:C ratio and supplementation of MSLP at 12 h. Increasing the R:C ratio and MSLP concentrations increased total volatile fatty acid (VFA) and propionic acid (C_3) concentrations while decreasing acetic acid (C_2) and butyric acid (C_4) concentrations; thus, the C_2:C_3 ratio was reduced. MSLP addition reduced protozoa and methanogen populations ($p < 0.05$). The calculated CH_4 production was decreased ($p < 0.05$) by the R:C ratios at 40:60 and supplementation of MSLP. Finally, the addition of MSLP as a phytonutrient may improve nutrient degradability and rumen fermentation properties while decreasing protozoa, methanogen population, and CH_4 production.

Keywords: phytonutrients; rumen fermentation; methane; *Mitragyna speciosa* Korth

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1. Introduction

Ruminants contribute to global warming by producing methane (CH_4), a significant greenhouse gas, as a byproduct of rumen microbial fermentation. Methane emissions cause energy loss in ruminants as well as contribute to greenhouse gas emissions [1,2]. Tropical plants are rich in phytonutrient compounds such as condensed tannins (CT) and saponins (SP), which might have antibacterial action, particularly in protozoal and methanogen populations. Phytonutrients influence gas production by altering microbial activities due to their ability to coat with fiber and protein content [3,4]. Cherdthong et al. [5] discovered that feeding 12 mg of *Delonix regia* seed pellet increased IVDM, NH_3-N, and C_3 percentage. Matra et al. [6] discovered that *Hylocereus undatus* peel powder, which contained CT and SP, reduced CH_4 production and protozoal population. *Garcinia mangostana* peels containing

CT have been demonstrated to be effective at mitigating CH_4 emissions in swamp buffaloes *(Bubalus bubalis)*, according to CT's action on rumen bacteria and methanogens. Moreover, SP has been shown to damage the membrane of rumen protozoa, reducing the amount of both protozoa and methanogenic archaea [7].

Mitragyna speciosa is a Southeast Asian natural medicinal plant that has been widely reported to be used to alleviate opioid dependence and withdrawal symptoms. *Mitragyna speciosa* Korth trees can grow to a height of 4–9 m and a width of 5 m. The tree grows in moist, humid, fertile soil that receives medium to full sun exposure. The leaves and smaller stems of the tree are consumed. Thai and Malaysians have traditionally used it to relieve pain, fatigue, and to treat opiate addiction [8]. *M. speciosa* was recently removed from Thailand's list of prohibited drugs. Those who own *M. speciosa*, on the other hand, will benefit from selling fresh leaves at higher prices, ranging from $8 to $10 per kilogram. *M. speciosa* leaves have been shown to have cocaine-like stimulating effects in low doses and opioid-like sedative narcotic effects in high doses [9]. Previous phytochemical research on *M. speciosa* discovered the presence of bioactive secondary metabolites from phytochemical categories such as total phenolic 407.83 ± 2.50 GAE mg/g (milligram of gallic acid equivalent per gram dry extract) and flavonoids 194.00 ± 5.00 QE mg/g (milligrams of quercetin equivalent per gram dry extract) and mitragynine content of 6.53–7.19% [10]. This plant has been shown in preclinical research to have antioxidant, antibacterial, antiproliferative, anti-inflammatory, and antinociceptive properties [11]. Despite its medical benefits, the use of *M. speciosa* has been linked to numerous cases of multiorgan toxicity and cardiotoxicity [9]. The roughage to concentrate ratio (R:C) in feeds is important for nutrient utilization throughout the production process. Concentrates in appropriate proportions can benefit fiber utilization by increasing the fermentable nutrients available to ruminal microorganisms [12]. However, the optimal R:C ratios in ruminants generally depend on the available nutrient content of each, relative costs, fermentation end products, and performance [13,14]. It was hypothesized that *M. speciosa* leaf powder (MSLP) could improve nutrient digestibility, rumen fermentation efficiency, microbial population, and CH_4 production. However, no research has been conducted on the effects of CT and SP in MSLP on the microbial population and CH_4 production in an in vitro gas production system.

Therefore, the objective of the research was to evaluate effects of the CT and SP from MSLP added to R:C ratio (60:40 or 40:60) on rumen fermentation, nutrient degradability, and CH_4 production in vitro.

2. Materials and Methods

2.1. Ethical Procedure

The study design and plan strictly followed the norms of the Animal Ethics Committee of Nakhon Phanom University in accordance with the Thailand Ethics of Animal Experimentation of the National Research Council (record No. U1-06878-2559).

2.2. Dietary Treatments and Experimental Design

The experimental design was randomly allocated according to a completely randomized design (CRD) in a 2 × 8 factorial arrangement, with three replicates per treatment, including blank triplicates (medium only). The first factor was two rations of R:C at 60:40 and 40:60, and the second factor was eight levels of MSLP addition (0, 1, 2, 3, 4, 5, 6, and 7% of the total substrate). The concentration of CT in total substrate were 0, 0.146, 0.292, 0.438, 0.584, 0.730, 0.876 and 1.022%, respectively, and concentration of SP in total substrate were 0, 0.121, 0.242, 0.363, 0.484, 0.605, 0.726, and 0.847%, respectively. The rice straw (*Oryza sativa* L.) (RS) was treated with 2.0% urea + 2.0% lime (ULTRS) by adding 2 g urea and 2 g lime in 100 mL of water to 100 g (91% DM). The concentrate was formulated with ingredients as shown in Table 1. The ULTRS and concentrate diet were dried at 60 °C and passed through a 1 mm screen to determine the chemical analysis of dry matter (DM), organic matter (OM), and crude protein (CP) [15]. The fiber content, especially neutral detergent fiber (NDF) and acid detergent fiber (ADF), was determined according to Van

Soest et al. [16]. Fresh *Mitragyna speciosa* Korth Havil leaves were purchased from a local market in Muang Nakhon Phanom, Nakhon Phanom, Thailand. The *M. speciosa* leaves were dried at 60 °C for 48 h and ground to pass through a 1-mm sieve (Cyclotech Mill, Tecator, Hoganas, Sweden). The CT and SP content were analyzed by using the modified vanillin-HCl method [17]. Mitragynine was isolated following the procedures described by Jamil et al. [18].

Table 1. Ingredients and chemical composition of concentrate, *Mitragyna speciosa* Korth leaves powder (MSLP) and 2% urea plus 2% calcium hydroxide ($Ca(OH)_2$) treated rice straw (ULTRS) used in the experiment.

Item	Concentrate	ULTRS	MSLP
Ingredient (kg of dry matter)			
Cassava chip	60.0		
Rice bran	13.5		
Coconut meal	13.0		
Palm kernel meal	6.0		
Urea	3.0		
Molasses	2.0		
Mineral premix	1.0		
Salt	1.0		
Sulfur	0.5		
Chemical composition			
Dry matter (%)	88.3	50.9	31.1
		–% Dry matter–	
Organic matter (OM)	92.6	90.4	94.3
Crude protein (CP)	14.2	5.6	21.2
Neutral detergent fiber (NDF)	19.1	70.2	51.4
Acid detergent fiber (ADF)	14.6	55.1	28.2
Condensed tannins	-	-	14.6
Saponins			12.1
Mitragynine			8.2

2.3. Rumen and Substrate Inocula

The rumen fluid was collected from two Thai native beef cattle with an initial body weight of 240 ± 10 kg. Thai native beef cattle were also adapted to concentrate diets (14.2% CP and 76.8% total digestive nutrient, dry-matter basis) (at 1.0% of live weight), and RS treated with urea and lime was fed ad libitum. Two cattle were housed in separate pens and received vitamin/mineral blocks and fresh water for 14 consecutive days. On day 15, before morning feeding, 1000 mL of rumen fluid was obtained from each beef cattle, using a suction pump, and then pooled and strained into an Erlenmeyer flask through four layers of cheesecloth. An experimental feed sample was weighed into 50 mL bottles of each total mixed substrate (200 mg). Bottles with the mixtures of substrate treatment received CO_2 flushing and were pre-warmed in a water bath at 39 °C for 96 h. Bottles were sealed with a rubber and aluminum cap and incubated for 96 h at 39 °C.

The medium was prepared for the determination of gas production and fermented material for various incubation durations. Ruminal fluid was mixed with reduced medium solution in a 2:1 (mL/mL) ratio, at 39 °C with continuous CO_2 flushing according to Makkar et al. [19]. A portion of the rumen-fluid medium mixture (40 mL) was transferred into each bottle, and blanks with only rumen fluid were used. They were then incubated at 39 °C in a water bath as described by Blummen and Orskov [20].

2.4. In Vitro Gas Production and Fermentation Characteristics

The production of gas was measured immediately following incubation and after at 0, 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h, as well as using a calibrated syringe and a pressure

transducer. Cumulative gas production data were fitted to the Ørskov and McDonald [21] model as follows:

$$y = a + b\ (1 - e^{-ct}) \quad (1)$$

where a = the gas production from the immediately soluble fraction (mL), b = the gas production from the insoluble fraction (mL), c = the gas production rate constant for the insoluble fraction (mL), t = incubation time (h), (a + b) = the potential extent of gas production (mL/h), and y = gas produced at time "t" (h).

Fermented liquid was collected at 4 and 8 h post-incubation to measure the pH, and then filtered through four layers of cheesecloth. Twenty-five milliliters of rumen fluid were separated into two portions; the first portion was for NH_3-N analysis and total volatile fatty acid (VFA) using 2 mL of sulphuric acid (H_2SO_4) added to 18 mL of incubation medium centrifuged at 16,000× *g* for 15 min, and the supernatant was stored at −20 °C before NH_3-N analysis (Kjeltech Auto 1030 Analyzer, Tecator, Sweden). Total volatile fatty acid (VFA) (mmol/L), and VFA profiles (%) [acetic acid (C_2), propionic acid (C_3), and butyric acid (C_4)] were analyzed, using high-performance liquid chromatography (instruments by controller water model 600E, water model 484 UV detector, column Nova-Pak C18, column size 3.9 mm × 300 mm, mobile phase 10 mM H_2PO_4, pH 2.5; Waters Corporation, Milford, Massachusetts, USA), and the production of CH_4 was estimated by using the Moss et al. [22] method as follows: CH_4 production (mmol/L) = 0.45 (C_2) − 0.275 (C_3) + 0.4 (C_4). The second portion of rumen fluid was preserved at −20 °C for extraction of deoxyribonucleic acid (DNA) according to Yu and Morrison [23]. The DNA was purified using columns from the QIAgen DNA Mini Stool Kit (QIAGEN, Valencia, CA, USA). Isolation of genomic DNA was used in real-time quantitative PCR assays with power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), forward and reverse primers, and template DNA. Specified primers to measure the microbial population of total bacteria according to Edwards et al. [24], protozoa [25], and methanogenic archaea [26]. The data of the microbial population was transferred to log10 prior to statistical analysis. The inocula after inoculation were filtered through pre-weighed Gooch crucibles, and residual dry matter was estimated. The percent loss in weight was determined and presented as in vitro dry matter degradability (IVDMD). The dried feed sample and residue left above were ashed at 550 °C, for determination of in vitro organic matter degradability (IVOMD), according to Tilley and Terry [27].

2.5. Statistical Analysis

All experimental data were analyzed as a 2 × 8 factorial arrangement in a completely randomized design (CRD) using SAS version 9.0 (SAS Inst. Inc., Cary, NC, USA) [28]. For all parameters, differences among treatment means were contrasted by Tukey's multiple comparison test when $p < 0.05$ [29].

3. Results and Discussions

3.1. Chemical Composition of Experimental Feeds

The nutritive value of the concentrate was 88.3, 92.6, 14.2, 19.1, and 14.6% for DM, OM, CP, NDF, and ADF, respectively (Table 1). The nutritive values of the MSLP were 94.3, 21.2, 51.4, and 28.2% for OM, CP, NDF, and ADF, respectively. The concentration of phytonutrients, especially CT, SP and mitraginine, in MSLP were 14.6, 12.1, and 8.2%, respectively, which were comparable to those reported by Goh et al. [10]; MSLP contained a total phenolic of 407.83 ± 2.50 GAE mg/g and flavonoids 194.00 ± 5.00 QE mg/g and mitragynine content of 6.53–7.19%. Moreover, CT and SP are high in MSLP and when compared to other tropical plants, they are found to be higher than in *Flemingia macrophylla*, containing 5.6% CT [30] and *Leucaena lucocephala*, containing 3.6% CT [31].

3.2. In Vitro Gas Production Kinetics

There was no interaction effect between R:C ratios and MSLP on the kinetics of gas ($p > 0.05$) (Table 2). Gas production kinetics, including gas production from the immediately

soluble fraction (a), the insoluble fraction (b), the gas production rate constant for the insoluble fraction (c), and the potential extent of gas production (a + b) and accumulated gas production (96 h), were increased ($p < 0.05$) by the R:C ratio at 40:60. It could be due to starch degradation is an essential role in regulating energy usage for rumen microbial growth, increasing rumen population, and feed digestion [12,13]. Furthermore, MSLP supplementation increased ($p < 0.05$) gas accumulation, which could be owing to the fact that stimulated the rumen microbe and increase the digestibility of incubated substrate, resulting in improved gas production kinetics. Due to the capability of CT and SP, the use of MSLP in diets influences microbial activity because of their ability to coat with fiber and protein [6]. Increasing the MSLP level higher than 5% in total substrate reduction could negatively affect the rumen microorganism activity, thus reducing the value of cumulative gas production at 96 h of incubation. This result is consistent with Cherdthong et al. [5] showed the highest level of CT and SP in pellets incorporating *Delonix regia* addition at 14 mg reduced cumulative gas production. Wanapat et al. [32] discovered that supplementing *Garcinia mangostana* peel, which contains CT at 20–60 g/kg DM, reduced total gas production.

Table 2. The effect of roughage-to-concentrate (R:C) ratio with *Mitragyna speciosa* Korth leaves powder (MSLP) supplementation on gas kinetics and cumulative gas production.

Treatment	R:C [1]	MSLP [2]	Gas Kenetics [3]				Gas (96 h) mL/0.2 g DM Substrate
			a	b	c	a + b	
1	60:40	0	−1.8	110.7	0.031	108.9	109.6
2		1	−1.9	113.5	0.032	111.6	111.4
3		2	−1.5	113.4	0.030	111.9	112.6
4		3	−2.0	114.6	0.034	112.6	115.8
5		4	−1.9	115.9	0.036	114.0	119.5
6		5	−2.1	116.2	0.038	114.1	118.8
7		6	−1.8	117.0	0.037	115.2	117.6
8		7	−1.9	116.9	0.035	115.0	117.0
9	40:60	0	1.0	122.8	0.051	123.9	125.1
10		1	1.3	121.2	0.052	122.5	124.3
11		2	1.4	123.0	0.054	124.4	125.6
12		3	1.2	125.2	0.053	126.4	128.8
13		4	1.6	131.1	0.054	132.7	133.4
14		5	1.4	131.9	0.056	133.3	135.7
15		6	1.4	127.4	0.055	128.8	120.4
16		7	1.6	127.6	0.055	129.2	124.5
	SEM		0.19	0.57	0.01	0.29	0.27
	Comparison						
	R:C		0.03	0.02	0.03	0.04	0.04
	MPLK		0.11	0.82	0.18	0.14	0.15
	Interaction		0.42	0.81	0.72	0.54	0.11

[1] R:C, roughage-to-concentrate ratio; [2] MPLK, *Mitragyna speciosa* Korth leaves powder (% of the total substrate on DM basis); [3] Gas kinetic, **a**. the gas production from the immediately soluble fraction (mL); **b**. the gas production from the insoluble fraction (mL); **c**, the gas production rate constant for the insoluble fraction (mL/h); **a + b**, the gas potential extent of gas production (mL); SEM, standard error of the mean.

3.3. In Vitro Digestibility

There was no significant interaction effect between the R:C ratio and MSLP addition on in vitro digestibility (Table 3). With an increasing concentrate proportion, the in vitro dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD) increased ($p < 0.05$). These findings agree with Viennasay et al. [33], regarding improving in vitro digestibility by increasing the proportion of concentrates in the diet. This could be due to the concurrent induction of microbial growth, which improved digestibility.

Table 3. The effect of roughage-to-concentrate (R:C) ratio with *Mitragyna speciosa* Korth leaves powder (MSLP) supplementation on rumen ecology and in vitro digestibility.

Treatment	R:C [1]	MSLP [2]	pH		NH_3-N (mg/dL)	In Vitro Digestibility, %			
			4 h	8 h		IVDMD 12 h	IVDMD 24 h	IVOMD 12 h	IVOMD 24 h
1	60:40	0	6.62	6.60	20.5	52.8	62.5	61.6	66.2
2		1	6.67	6.62	18.4	53.9	63.1	64.8	68.7
3		2	6.71	6.65	18.4	54.6	64.2	65.3	69.5
4		3	6.62	6.61	18.1	55.8	65.1	66.4	71.1
5		4	6.65	6.62	17.4	57.6	68.8	73.1	78.2
6		5	6.64	6.61	17.2	56.9	67.1	72.8	77.9
7		6	6.66	6.62	17.1	57.5	67.2	71.0	76.0
8		7	6.65	6.60	16.0	56.9	67.0	70.1	75.9
9	40:60	0	6.60	6.58	24.8	55.8	66.1	68.1	73.4
10		1	6.64	6.61	23.0	58.0	68.3	72.5	77.0
11		2	6.61	6.59	22.8	58.9	69.1	73.3	78.5
12		3	6.66	6.62	22.5	61.8	70.6	74.9	79.6
13		4	6.65	6.61	20.1	63.7	74.2	78.8	83.4
14		5	6.61	6.59	19.8	63.8	74.1	78.1	82.5
15		6	6.61	6.58	19.0	63.0	73.6	77.4	82.1
16		7	6.60	6.59	18.1	62.9	73.5	76.9	81.0
	SEM		0.38	0.57	0.77	1.97	2.43	1.85	2.47
	Comparison								
	R:C		0.05	0.05	0.04	0.01	0.01	0.01	0.002
	MPLK		0.06	0.05	0.04	0.02	0.01	0.01	0.01
	Interaction		0.08	0.16	0.45	0.52	0.71	0.53	0.68

[1] R:C, roughage-to-concentrate ratio; [2] MPLK, *Mitragyna speciosa* Korth leaves powder (% of the total substrate on DM basis); NH_3-N, ammonia–nitrogen; pH4, pH at 4 h after incubation; pH 8, pH at 8 h after incubation; IVDMD 12 h, in vitro dry matter digestibility at 12 h after incubation; IVDMD 24 h, in vitro dry matter digestibility at 24 h after incubation; IVOMD 12 h, in vitro organic matter digestibility at 12 h after incubation; IVOMD 24 h, in vitro organic matter digestibility at 24 h after incubation; SEM, standard error of the mean.

The degradability of IVDMD and IVOMD has also increased with the supplementation of MSLP. Maximum IVDMD and IVOMD were achieved 24 h after incubation at a R:C ratio of 40:60 and 4% MSLP supplementation, which were 12.3% and 13.6% higher than the control, respectively, resulting in improved cumulative gas production. Similarly, Matra et al. [6] showed that adding *Hylocereus undatus* peel powder containing CT and SP increased the mean IVDMD and IVOMD values. According to Gunun et al. [34], supplementing *Nephelium lappaceum* L. peel powder with CT enhanced their in vitro degradability. It is possible that the CT concentration of MSLP lowers protozoa numbers and bacteria are protozoa's feed substrate; thus, it would be assumed that the population of fibrolytic bacteria would increase [1,7,35]. In contrast, Cielak et al. [36] discovered that CT decreased nutritional digestibility, but only at the higher dose of 100 mg of *Sanguisorba officinalis* supplementation. Moreover, CT and SP had an inhibiting effect on nutrient digestion by reducing the number of ruminal fibrolytic bacteria [37]. Tannins can form complexes with proteins and carbohydrates via hydrogen or hydrophobic interactions, or both, reducing their availability to microbial breakdown and fermentation [1,2].

3.4. Ruminal pH and Ammonia-Nitrogen (NH_3-N) Concentration

There were no interactions between the R:C ratio and MSLP addition on ruminal pH and the NH_3-N concentration (Table 4). The pH value ranged from 6.58 to 6.71 for all treatments. According to Zicarelli et al. [38], ruminal pH of 6.23–6.69 has been reported to maintain the normal activity of cellulolytic bacteria. Strategic CT and SP containing feedstuffs addition may improve rumen efficiency by maintaining higher pH and promoting microbial protein synthesis [31,33]. MSLP supplementation lowered ruminal NH_3-N concentration ($p < 0.01$) due to CT and SP in MSLP, which protected the diet's protein

degradability. This result agreed with Wanapat et al. [32] and Patra et al. [3] who showed that CT had nutritional benefits by generating a protein–tannin complex, lowering the availability of ruminal degradation feed protein and the production of NH_3-N. In contrast, Matra et al. [6], discovered that increasing *Hylocereus undatus* peel supplementation increased mean NH_3-N concentration values. In the phytonutrient supplementation groups, the rumen NH_3-N concentration increased [35]. Furthermore, due to tannin binding, more protein was available to be rendered inaccessible and reduced to NH_3-N by the bacteria.

Table 4. The effect of roughage-to-concentrate (R:C) ratio with *Mitragyna speciosa* Korth leaves powder (MSLP) supplementation on in vitro total volatile fatty acids (VFA), VFA profiles, and methane (CH_4) production.

Treatment	R:C [1]	MPLK [2]	Total VFA, (mM/L)	C_2, (%)	C_3, (%)	C_4, (%)	C_2:C_3 Ratio	CH_4 Production [3], mM
1	60:40	0	40.1	69.8	20.1	10.1	3.5	29.9
2		1	41.3	69.6	21.3	9.1	3.3	29.1
3		2	41.9	68.7	22.8	8.5	3.0	28.0
4		3	42.0	66.4	23.1	10.5	2.9	27.7
5		4	43.9	65.2	25.6	9.2	2.5	26.0
6		5	43.7	65.9	25.7	8.4	2.6	25.9
7		6	43.1	65.8	25.4	8.8	2.6	26.1
8		7	42.9	61.6	25.5	12.9	2.4	25.9
9	40:60	0	43.2	67.8	22.5	9.7	3.0	28.2
10		1	44.9	66.7	23.1	10.2	2.9	27.7
11		2	45.7	66.4	23.5	10.1	2.8	27.5
12		3	46.0	65.1	24.2	10.7	2.7	26.9
13		4	49.7	60.1	26.8	13.1	2.2	24.9
14		5	49.4	60.9	26.9	12.2	2.3	24.9
15		6	48.5	60.3	26.7	13.0	2.3	25.0
16		7	48.3	58.7	27.1	14.2	2.2	24.6
	SEM		2.04	0.75	0.04	0.32	0.08	0.13
	R:C		0.01	0.001	0.001	0.002	0.005	0.004
	MPLK		0.04	0.03	0.03	0.76	0.04	0.04
	Interaction		0.26	0.41	0.53	0.82	0.12	0.47

[1] R:C, roughage-to-concentrate ratio; [2] MPLK, *Mitragyna speciosa* Korth leaves powder (% of the total substrate on DM basis); C_2, acetic acid; C_3, propionic acid; C_4, butyric acid; [3] calculated according to Moss et al. [20], CH_4 production = 0.45 (acetic acid) − 0.275 (propionic acid) + 0.4 (butyric acid); SEM, standard error of the mean.

However, the result showed that ruminal pH at 8 h after incubation was reduced with an increased proportion of concentrates in the diet, whereas it was lowest when the R:C ratio was at 40:60. Additionally, Wanapat et al. [14], and Phesatcha et al. [13], observed that meals high in concentrate induced a significant decrease in ruminal pH, inhibited digestion rate, and decreased cellulolytic bacteria activity.

3.5. Volatile Fatty Acid and Methane Production

There was no interaction effect between the R:C ratio and the addition of MSLP on overall VFA and VFA profiles (Table 4). The total concentrations of VFA ranged from 40.1 to 49.7 mM, which was similar to the findings of Kang et al. [39] and Viennasay et al. [33]. The R:C ratio at 40:60 increased total VFA and C_3, but lowered C_2 and the C_2:C_3 ratios ($p < 0.05$). When feed degradability was increased, total VFA and the proportion of C_3 increased, while the proportion of C_2 to C_3 decreased [5,6,13,33]. This could be because a concentrate contains a portion of highly degradable carbohydrates, such as cassava chips. A high starch concentrate diet tended to ferment toward C_3, in which rumen bacteria digested the soluble carbohydrate and starch in order to increase total VFA and C_2, C_3, and C_4 in their cells [12].

In the current study, TVFA and C_3 increased ($p < 0.05$) with the addition of MPLK at 4% of total substrate, compared to the control by 15.05% and 19.1%, respectively. The expected shift in the VFA profile from C_2 to C_3 ($p < 0.05$) was related to the H_2 shift from

CH_4 production, which is nutritionally advantageous for beef cattle. In the current study, adding MPLK to the diet significantly reduced CH_4 production. Several studies have found that the addition of plant-based phytonutrients (CT and SP) to ruminants can reduce CH_4 production; *Garcinia mangostana* peel [32,40], *Flemingia macrophylla* [31], *Nephelium lappacuem* peel [34], *Delonix regia* [5], *Leucaena lucocephala* [31]. Cieslak et al. [36] stated that CO_2 and H_2 were converted to CH_4 by methanogens such as *Methanopyrales*, *Methanocellales* and *Methanomicrobiales*. As a result, reduced CH_4 moderation possibilities that also ensure efficient energy use are required.

3.6. Rumen Microorganism

There was no interaction between the R:C ratio and MSLP addition (Table 5). The total bacteria and protozoal population increased ($p < 0.05$) as the proportion of concentrates in the diet increased. The number of protozoa in the rumen fluid increased with high-concentrate feeding, protozoa appear to have a buffering effect due to their rapid ingestion of starch granules, which slows ruminal bacteria fermentation and the subsequent production of organic acids [2,41]. This buffering effect tends to stabilize ruminal fermentation and lower the redox potential of rumen digesta [1,3]. This is similar to the findings of Cherdthong et al. [5], who indicated that the synthesis of ruminal microbial bacteria is dependent on an adequate supply of glucose for energy and NH_3-N for peptide bond formation. According to Suriyapha et al. [12] and Phesatcha et al. [13], a high-level concentrate diet significantly increased the total bacteria in the rumen.

Table 5. The effect of roughage-to-concentrate (R:C) ratio with *Mitragyna speciosa* Korth leaves powder (MSLP) supplementation on microbial population.

Treatment	R:C [1]	MPLK [2]	Total Bacteria ($\times 10^{10}$ cells/mL)		Protozoa ($\times 10^{5}$ cells/mL)		Methanogens ($\times 10^{3}$ cells/mL)	
			4 h	8 h	4 h	8 h	4 h	8 h
1	60:40	0	7.4	15.5	5.1	6.9	6.9	8.6
2		1	8.2	19.3	4.9	6.5	6.5	8.5
3		2	9.1	21.8	4.5	6.2	5.7	7.9
4		3	10.4	22.5	4.0	6.1	5.4	7.5
5		4	11.6	24.4	3.0	4.9	4.1	5.6
6		5	11.0	24.1	2.9	4.3	3.8	5.5
7		6	10.7	24.3	2.5	4.1	3.6	5.0
8		7	10.3	23.9	2.4	3.3	3.5	4.1
9	40:60	0	12.1	23.8	6.6	7.4	4.8	5.9
10		1	12.8	25.4	6.3	7.2	4.7	5.1
11		2	13.2	26.5	6.1	6.9	3.4	4.9
12		3	13.9	27.1	6.0	6.5	3.3	4.7
13		4	15.4	29.8	5.1	5.4	2.1	3.2
14		5	16.8	29.5	5.0	5.3	2.0	3.1
15		6	16.5	29.4	4.9	5.0	1.8	2.6
16		7	16.0	28.7	4.5	5.1	1.7	2.6
SEM			0.95	0.87	0.28	0.53	0.62	0.70
	Comparison							
	RC		0.001	0.001	0.001	0.003	0.04	0.01
	MPLK		0.005	0.002	0.006	0.002	0.04	0.01
	Interaction		0.38	0.43	0.45	0.69	0.94	0.38

[1] R:C, roughage-to-concentrate ratio; [2] MPLK, *Mitragyna speciosa* Korth leaves powder (% of the total substrate on DM basis); SEM, standard error of the mean.

Furthermore, MPLK supplementation reduced the number of protozoa and methanogen archaea ($p < 0.05$) and CH_4 production in the rumen, which could be attributed to the availability of phytonutrients such as CT and SP in the MSLP. Ku-Vera et al. [7] found CT and SP reduced the production of protozoa, methanogens, and CH_4. The CT has a direct effect on rumen methanogenic archaea by binding the proteinaceous adhesin or parts of the

cell envelope, preventing the formation of the methanogen–protozoa complex, decreasing interspecies hydrogen transfer, and inhibiting methanogen growth [42,43]. Moreover, Vasta et al. [1] proposed that SP could disrupt protozoa by forming complexes with sterols on the protozoal membrane surface, causing the membrane to become impaired and disintegrate. Furthermore, some SP influence membrane proteins such as Ca^{2+} channels and Na^{+} and K^{+} ATPases [44]. Because some methanogens live in symbiotic relationships with protozoa and because protozoa provide hydrogen as a substrate for CH_4 formation, any decrease in the protozoa population may reduce the methanogen population and methanogenesis [45–47]. Furthermore, CTs act as H_2 sinks, reducing their availability for CO_2 reduction to CH_4, as hydrogen was shifted from CH_4 to C_3 [1,2,48]. Kang et al. [39] found that supplementing *Flemingia macrophylla* leaf meal improved in vitro fermentation, and lowered CH_4. According to Polyorach et al. [40], reveled adding *Garcinia mangostana* peel which contains CT and SP reduced the population of protozoa and methanogens and lowered CH_4 production in swamp buffaloes.

Acacia cyanophylla supplemented at 60% and 30% reduced 37.5 and 56.2 percent of CH_4 production, respectively, due to the high CT content that reduced methanogen populations [42].

4. Conclusions and Recommendations

In this experiment, the results revealed an R: C ratio of 40:60 together with MSLP content of 4% in total substrate (CT 0.584% and SP 0.484%), enhanced rumen fermentation, and mitigated CH_4 production. Therefore, MSLP has the potential to increase ruminant productivity and reduce the associated methane emissions.

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