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RESEARCH ARTICLE
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Microencapsulated-Bioactive Compounds from Medicinal Leaf Extracts Used as Feed Supplements: Effects on *In Vitro* Rumen Fermentation, Microbial Population and Methane Mitigation

Maharach Matra [1], Srisan Phupaboon [2], Pajaree Totakul [3], Ronnachai Prommachart [4] and Metha Wanapat [2,*]

[1]Division of Animal Science, Department of Agricultural Technology, Faculty of Technology, Mahasarakham University, Maha Sarakham 44150, Thailand
[2]Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand
[3]Division of Animal Science, Faculty of Agricultural Technology, Rajamangala University of Technology Thanyaburi, Pathum Thani 12130, Thailand
[4]Department of Animal Science, Faculty of Agriculture and Natural Resources, Rajamangala University of Technology, Tawan-Ok 20110, Thailand
*Corresponding author: metha@kku.ac.th

ABSTRACT

This investigation aimed to assess the effects of microencapsulated-Marijuana leaf extracts (MMALE) supplementation on gas production kinetics, rumen fermentation, microbial populations, and methane production. A Completely randomized design (CRD) was used to randomly assign respective treatments. Results showed that *in vitro* dry matter degradability was improved with MMALE supplement at 6% total DM substate. The ammonia-nitrogen concentrations were significantly different among the four groups. Total volatile fatty acids (VFA) and propionate production were increased, while acetate to propionate ratio, butyrate, and methane production were reduced when compared to the control group. Inclusion with MMALE significantly enhanced the cellulolytic bacteria, while *Methanobacteriales* was decreased. Therefore, MMALE is a promising plant-based bioactive substrate that could be utilized to modulate rumen fermentation in the diet.

Keywords: Marijuana, Medicinal plant, Rumen fermentation, Bioactive compounds, Microencapsulation

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INTRODUCTION

Currently, the potential to modulate rumen fermentation characteristics using medicinal plants (MP) as an alternative to antimicrobials is gaining popularity (Gupta and Birdi, 2017). MP includes a diverse range of bioactive compounds (BCs) that possess antioxidant, anti-inflammatory, and antibacterial properties (Valenzuela-Grijalva et al., 2017). More specifically, the six groups of BC found in plant-based functional foods include alkaloids, flavonoids, phenolics, polysaccharides, saponins, and others (Jiang et al., 2020). Accordingly, Petrič et al. (2020), Huang et al. (2021) and Chanjula et al. (2022) reported that the medicinal plant's dietary substrate had a high level of ruminal antioxidant capacity, the capacity to improve rumen fermentation and reduce methane generation.

Cannabis plant is one of the MPs used by humans and has also been utilized to produce oil, fiber and feed additives for food products (Farag and Kayser, 2017). Among the 545 identified compounds in cannabis, which is a complex medicinal plant with several BC classes, there are at least 11 steroids, 26 flavonoids, 104 cannabinoids, and 120 terpenoids (ElSohly and Gul, 2014; Pollastro et al., 2018). A typical instance of this is the MP, Marijuana (*Cannabis indica* L.) is an annual plant species in the family Cannabaceae, which produces large amounts of BC. They are produced for a variety of uses, including BC compound extraction for medicinal purposes (Punja, 2021). Cannabis has inspired a current wave of interest due to its numerous potential medicinal benefits as an aid to digestion and

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appetite stimulation (Zuardi, 2006) and as an analgesic, possibly as a supplement to or a replacement for opiates in the treatment of chronic pain (Lucas, 2012). Nevertheless, no previous study has elucidated BC extracts protection by using microencapsulation technology from Marijuana leaf as a feed additive to enhance ruminal fermentation-end products in ruminants. Furthermore, microencapsulation is the method of enveloping one substance within another substance on a very small scale (less than one micron to several hundred microns) (Jyothi et al., 2012). They are a new technique that is increasingly being applied in animal feeding to provide stable products.

Thus, the objective of this study was to determine the optimal level of MMALE on nutrient degradation, rumen fermentation, and microbial population by using an *in vitro* fermentation assay.

MATERIALS & METHODS

Animals

The Institute of Animals for Scientific Purpose Development (IAD), Thailand approved all animal procedures (approval number: U1-06878-2560). Four Holstein-crossbred dairy cows, with an initial body weight of 400±10kg, were selected as donors of rumen fluid. The animals were fed twice daily at 07:00 and 16:00 o'clock a total mixed ration *ad libitum*, with free access to a mineral block and clean water.

MMALE Preparation

Marijuana leaf was dried and ground to a sieve size of 1mm. The water and powder were mixed together and subjected to microwave irradiation at a temperature of 60°C, with a voltage of 100 volts, for a duration of 35 minutes. Afterwards, the resulting mixture was filtered to separate the liquid components. The liquid was mixed with tween 80 and chitosan (Nouri, 2019), and then a Bŭchi B-191 Mini Spray-Dryer was used to spray-dry the sample (Kurek et al., 2020).

Chemical Analyses

The results of the chemical analyses are shown in Table 1. The concentrate, roughage, and MMALE were analyzed to determine their crude protein (method number 984.13), dry matter (method number 967.03), and organic matter (method number 942.05) content, following the guidelines provided by the AOAC (2012; method number 973.18). The fiber contents were analyzed using the Ankom A200i Fibre Analyser (Ankom Technology Co., New York, USA), following the methods described by Van Soest et al. (1991). MMALE and Marijuana leaf meal (MLM) were analyzed for bioactive compounds (total flavonoid and phenolic compounds) and antioxidant capacities (2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid); ABTS, 2, 2-diphenyl-1-picrylhydrazyl; DPPH, and ferric reducing antioxidant power; FRAP capacity). Further elucidation of these terms can be found in the study of Phupaboon et al. (2022).

Experimental Design and Treatments

Four dietary treatments were assigned according to a completely randomized design (CRD). MMALE was supplemented to the treatments at various levels (0, 4, 6, and 8% of the total substrate).

In vitro Rumen Fermentation Preparation and Procedures

Under continuous CO_2 flushing, the rumen fluid and medium solution (1:2, v:v) were combined. Firstly, rumen fluid samples were collected by entering a tube attached to a vacuum pump through the mouth to the midpoint of

Table 1: Chemical composition of feed used in the experiment

Items	Concentrate	Rice straw	MLM	MMALE
Ingredients (% as fed)				
Cassava chip	54.0			
Rice bran meal	17.0			
Palm kernel meal	13.0			
Soybean meal	10.5			
Urea	2.5			
Sulphur	1.0			
Salt	1.0			
Mineral mixed[1]	1.0			
Chemical composition				
Dry matter (DM, %)	90.5	89.4	92.6	88.5
		----------% dry matter----------		
Organic matter (OM)	92.2	85.4	86.1	93.9
Crude protein (CP)	14.6	2.4	19.1	20.5
Neutral-detergent fiber (NDF)	20.5	78.9	45.2	70.7
Acid-detergent fiber (ADF)	8.2	52.6	26.4	23.1
Phytonutrient compounds				
TPC (mg GAE/g DM)	-	-	218.9	266.1
TFC (mg QUE/g DM)	-	-	88.6	69.8
Antioxidant capacity				
DPPH (%)	-	-	39.3	83.5
ABTS (%)	-	-	94.6	84.3
FRAP (mg TROE/g DM)	-	-	23.7	30.9

MLM, Marijuana leaf meal; MMALE, microencapsulated-Marijuana leaf extracts; TPC, total phenolic content; TFC, total flavonoid content; DPPH, 2, 2-diphenyl-1-picrylhydrazyl as DPPH radical scavenging activity; ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) as ABTS radical scavenging activity; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; QUE, quercetin equivalent; TROE, trolox equivalent; [1] Mineral premix (contains per kg): vitamin A 10,000,000IU; vitamin D 1,600,000IU; vitamin E 70,000IU; Fe 50g; Mn 40g; Zn 40g; Cu 10g; I 0.5g; Se 0.1g; Co 0.1g

the rumen and into a plastic flask. Then, the samples were placed into a bottle equipped with thermal insulation set at 39°C. Secondly, the medium solution preparation consists of 950mL of distilled water, 480mL of buffer solution, 99mL of freshly prepared reduction, 2.44mL of resazurine, 480mL of macro-mineral, and 0.24mL of micro-mineral solution prepared per 2,000mL, as more detailed in Matra et al. (2021). Dietary substrates were measured into glass bottles (60mL), then after incubating the bottles at a temperature of 39°C, 40mL of rumen inocula were added and sealed with rubber stoppers and aluminum caps.

Gas production was measured after 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96h of incubation. Incubation samples were kept at 12, 24, and 48h and processed for pH value. The samples were centrifuged at a force of 16,000 × g for 15 minutes, following filtration through cheesecloth, then to analyze the volatile fatty acids (HPLC, ETL Testing Laboratory, Inc., Cortland, USA; Samuel et al., 1997), ammonia-nitrogen concentration (micro-Kjeldahl methods; AOAC, 2012), methane production (GC machine, Shimadzu Co Ltd., Kyoto, Japan), and dry matter, filter bags were weighed for determination of *in vitro* nutrient degradability.

DNA Extraction and Determination

Total genomic DNA (gDNA) was extracted from rumen fluid at 1mL (12, 24, and 48h incubation) by using the QIAamp Fast DNA Stool Mini kit (Qiagen, Hilden, Germany). Using a Nanodrop spectrophotometer (Thermo Scientific, USA), the absorbance at OD260/280 = 1.8 to 2.0 indicated the quality of the gDNA. Real-time PCR was used to identify key microbial groups, namely *Fibrobactor succinnogenes, Ruminococcus flavefaciens, Ruminococcus albus, Megasphaera elsdenii, Butyribrivio fibrisolvens,* and *Methanobacteriales* using specific primers, as presented in Table 2. Polymerase chain reaction was conducted for amplification and detection (Maxima SYBR Green qPCR Master Mix, BioRad, Hercules, CA, USA). Additional information regarding the methodology can be found in the publication by Koike and Kobayashi (2001).

Statistical Methods

The data were analyzed as a Completely randomized design (CRD) using the GLM procedure of SAS (2013). Tukey's test was used to compare the experimental treatment means. Treatment means differences $P<0.05$ and $P<0.01$ were indicated as significantly different. Orthogonal polynomial contrasts were analyzed to determine whether the effect of MMALE level was linear, quadratic, or cubic.

RESULTS

Gas Production Kinetics and Nutrient Degradability

Table 3 presents the impact of MMALE supplementation on gas production kinetics and nutrient degradability. Gas production including cumulative gas production, the gas production from the insoluble fraction (b), the gas production rate constant for the insoluble fraction (c) and the potential extent of gas production (a+b) were quadratically different ($P<0.05$) with MMALE supplementation, while the gas production from the immediately soluble fraction (a) did not differ ($P>0.05$) in the four treatments. Moreover, at 12, 24, and 48h in vitro DM degradability increased significantly ($P<0.05$), especially with MMALE at 6% of the total DM substrate having the highest value.

Table 2: The specific primers of microbes in the rumen

Species	Specific primers	Primer sequences (5'-3')	PCR products (bp)	References
Fibrobacter succinogenes	Fs219f	GGTATGGGATGAGCTTGC	446	Koike and Kobayashi (2001)
	Fs654r	GCCTGCCCCTGAACTATC		
Ruminococcus albus	Ra1281f	CCCTAAAAGCAGTCTTAGTTCG	175	
	Ra1439r	CCTCCTTGCGGTTAGAACA		
Ruminococcus flavefaciens	Rf154f	TCTGGA AACGGATGG TA	295	
	Rf425r	CCTTTAAGACAGGAGTTTACAA		
Megasphaera elsdenii	Mef	GACCGAAACTGCGATGCTAGA	128	Ouwerkerk et al. (2002)
	Mer	TCCAGAAAGCCGCTTTCGCCACT		
Butyrivibrio fibrisolvens	Bff	CGCATGATGCAGTGTGAAAAGCTC	625	Fernando et al. (2010)
	Bfr	CCTCCCGACACCTATTATTCATCG		
Methanobacteriales	Mbt857f	GGGCTTGCTTTGGAAACTGTT	343	Yu et al. (2005)
	Mbt1196r	CCCACCGATGTTCCTCCTAA		

Table 3: Supplementation of microencapsulated-Marijuana leaf extracts on gas kinetics and nutrient degradability

Treatment	MMALE	Gas kinetics[1]				Cumulative gas[2] at 96h	IVDMD (% DM)		
		a	b	c	a+b		12h	24h	48h
1	0	-2.0	97.1^{a}	0.035^{a}	95.1^{a}	95.5^{a}	54.1^{a}	59.0^{a}	65.0^{a}
2	4	-1.8	98.8^{b}	0.040^{b}	97.0^{b}	97.4^{b}	56.3^{b}	63.1^{b}	67.8^{b}
3	6	-1.8	107.2^{c}	0.047^{c}	105.4^{c}	105.8^{c}	60.7^{c}	65.3^{c}	70.1^{c}
4	8	-2.1	97.5^{a}	0.038^{d}	95.4^{a}	95.7^{a}	55.4^{a}	61.3^{a}	66.0^{a}
SEM		0.56	1.12	0.02	1.56	1.38	0.91	0.97	1.53
Orthogonal polynomials									
Linear		0.12	0.06	0.08	0.15	0.12	0.22	0.25	0.67
Quadratic		0.08	<0.01	0.03	0.01	<0.01	0.03	0.04	0.04
Cubic		0.34	0.64	0.11	0.78	0.60	0.35	0.29	0.98

MMALE, microencapsulated-Marijuana leaf extracts (% of total DM substrate); IVDMD, *in vitro* dry matter degradability; SEM, standard error of mean; [1] Gas production kinetics, 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 potential extent of gas production (mL); [2] Cumulative gasses at 96h (mL/0.2g DM substrate); [a-d] Means within the same column with different letters are significantly different at $P<0.05$.

In vitro Fermentation Parameters

The pH (12, 24, and 48h) of the rumen fluid was similar (P>0.05) among treatments, as the incubation time increased, the pH slightly decreased. Whilst ammonia-nitrogen concentration (12, 24, and 48h) was greater (P<0.01) in the inoculum with an increase in MMALE level at 6% than 8% of total DM substrate (Table 4). The concentration of acetate and acetate to propionate ratio were quadratically reduced (P<0.05) with addition of MMALE to the substrate. Total VFA and propionate production were enhanced (P<0.05) and the greatest concentration were achieved at 6% of the total DM substrate, however there was no significant influence (P>0.05) on butyrate content. Methane concentrations at 12, 24, and 48h of incubation were significantly reduced (linear effect; P<0.05) by the MMALE supplementation. The effects of the treatment supplements on volatile fatty acid and methane production are presented in Table 5.

Ruminal Microbial Population

As shown in Table 6, the populations of cellulolytic bacteria namely *Fibrobactor succinnogenes, Ruminococcus albus* and*, Ruminococcus flavefaciens* were significantly affected (P<0.05) by increasing MMALE levels (24 and 48h), but the values at 12h had not been influenced (P>0.05). Increased numbers at 24 and 48h were seen with additional levels of MMALE for *Butyribrivio fibrisolvens* (cubic; P<0.05 and linear; P<0.05), with highest values for the treatments with MMALE at 6% of the total DM substrate. Importantly, the abundance of *Methanobacteriales* at 12, 24, and 48h showed a significant linear reduction (P<0.05) with increasing levels of MMALE.

Table 4: Supplementation of microencapsulated-Marijuana leaf extracts on ruminal pH and ammonia-nitrogen concentration

Treatment	MMALE	pH			Ammonia-nitrogen (mg/dL)		
		12h	24h	48h	12h	24h	48h
1	0	6.84	6.80	6.78	10.4^{a}	11.2^{a}	13.8^{a}
2	4	6.82	6.82	6.80	12.7^{b}	12.4^{b}	15.6^{b}
3	6	6.84	6.83	6.79	13.3^{c}	13.5^{c}	16.5^{c}
4	8	6.87	6.83	6.82	9.4^{d}	10.2^{d}	11.8^{d}
SEM		0.02	0.01	0.02	0.18	0.15	0.23
Orthogonal polynomials							
Linear		0.16	0.09	0.17	0.35	0.58	0.76
Quadratic		0.22	0.31	0.56	<0.01	0.01	<0.01
Cubic		0.53	0.83	1.03	0.18	0.24	0.46

MMALE, microencapsulated-Marijuana leaf extracts (% of total DM substrate); SEM, standard error of mean; $^{a-d}$ Means within the same column with different letters are significantly different at P<0.05.

Table 5: Supplementation of microencapsulated-Marijuana leaf extracts on volatile fatty acids and methane production

Treatment	MMALE	VFA (mol/100mL)			C_2:C_3	Total VFA (mmol/L)	Methane production (%)		
		C_2	C_3	C_4			12h	24h	48h
1	0	70.7^{a}	22.7^{a}	6.6	3.11^{a}	65.9^{a}	26.1^{a}	29.4^{a}	33.5^{a}
2	4	69.8^{b}	23.9^{b}	6.3	2.92^{b}	72.6^{b}	25.2^{b}	28.5^{b}	32.6^{b}
3	6	67.3^{c}	25.9^{c}	6.8	2.60^{c}	83.3^{c}	23.7^{c}	27.0^{c}	32.1^{c}
4	8	70.5^{a}	23.5^{b}	6.0	3.00^{a}	70.2^{b}	23.5^{c}	26.5^{c}	31.9^{c}
SEM		0.42	0.35	0.52	0.07	1.85	0.08	0.06	0.07
Orthogonal polynomials									
Linear		0.34	0.46	0.75	0.47	0.32	0.03	0.02	0.01
Quadratic		0.03	0.02	0.18	0.04	0.04	0.73	0.51	0.54
Cubic		0.24	0.13	0.13	0.28	0.53	0.52	0.42	0.48

MMALE, microencapsulated-Marijuana leaf extracts (% of total DM substrate); VFA, volatile fatty acids; C_2, acetate; C_3, propionate; C_4, butyrate; C_2:C_3, acetate to propionate ratio; SEM, standard error of mean; $^{a-c}$ Means within the same column with different letters are significantly different at P<0.05.

Table 6: Supplementation of microencapsulated-Marijuana leaf extracts on rumen microbial population

Species	Incubation time	MMALE				SEM	Orthogonal polynomials		
		0	4	6	8		L	Q	C
Fibrobacter succinogenes	12h	0.9	0.4	0.3	0.2	0.76	0.56	0.59	0.33
(×10^6 copies/mL)	24h	2.1^{a}	0.6^{b}	0.5^{b}	0.1^{b}	0.68	<0.01	0.02	0.34
	48h	2.3^{a}	0.8^{b}	0.9^{b}	0.3^{b}	0.27	<0.01	0.01	0.01
Ruminococcus albus	12h	0.8	0.3	0.4	0.4	0.37	0.20	0.21	0.36
(×10^8 copies/mL)	24h	1.8^{a}	0.5^{b}	0.4^{b}	0.3^{b}	1.03	<0.01	<0.01	0.02
	48h	2.0^{a}	1.4^{b}	1.4^{b}	1.2^{b}	0.24	0.01	0.20	0.21
Ruminococcus flavefaciens	12h	0.5	0.7	0.8	0.6	0.45	0.68	0.35	0.83
(×10^7 copies/mL)	24h	1.9^{a}	0.4^{b}	0.5^{b}	0.4^{b}	0.31	<0.01	0.01	0.07
	48h	2.0^{a}	0.7^{b}	0.6^{b}	0.5^{b}	0.29	<0.01	0.01	0.12
Megasphaera elsdenii	12h	0.8	0.9	1.0	0.9	0.23	0.43	0.35	0.44
(×10^7 copies/mL)	24h	1.4^{a}	1.2^{b}	1.2^{b}	0.8^{c}	0.19	0.01	0.53	0.17
	48h	0.9	0.9	0.9	0.7	1.34	0.18	0.25	0.13
Butyrivibrio fibrisolvens	12h	1.0	1.1	1.5	1.3	0.33	0.15	0.51	0.28
(×10^6 copies/mL)	24h	2.0^{a}	1.7^{a}	3.6^{b}	1.8^{a}	0.79	0.57	0.12	0.02
	48h	2.4^{a}	3.2^{b}	3.7^{c}	4.1^{c}	0.60	0.02	0.57	0.07
Methanobacteriales	12h	1.2^{a}	0.9^{b}	0.6^{c}	0.5^{c}	0.14	<0.01	0.19	0.65
(×10^7 copies/mL)	24h	1.5	1.1	1.0	1.0	0.27	0.04	0.21	0.69
	48h	2.6^{a}	2.2^{b}	1.8^{c}	1.4^{d}	0.24	<0.01	0.92	0.74

MMALE, microencapsulated-Marijuana leaf extracts (% of total DM substrate); SEM, standard error of mean; L, linear; Q, quadratic; C, cubic; $^{a-d}$ Means within the same row with different letters are significantly different at P<0.05.

DISCUSSION

Gas Production Kinetics and Nutrient Degradability

Gas production mostly depends on the substrates' capacity to degrade soluble components and the process of breaking down fermented substances into volatile fatty acids and the production of microbial biomass (Elghandour et al., 2017). Other factors including BCs affect the production of gases (Bureenok et al., 2019). The present study shows that gas production was enhanced with MMALE addition. It may be suggested that BC may coat in protein and fiber particles, and that the inclusion of MMALE containing BC in treatment substrates affects microbial activity. Moreover, it could be that the regulation of energy use for rumen microbial growth depends on the efficient breakdown of starch, improving feed digestion and rumen diversity (Phesatcha et al., 2020). In this trial, MMALE supplementation resulted in an improvement of *in vitro* dry matter degradability. This might be as a result of an increase in microbes (BC were supplied by MMALE to the ruminal microbial activity), which may enrich nutrient degradability. The cellulolytic bacteria that can be grown that are primarily responsible for digesting fiber include *F. succinogenes, R. albus, R. flavefaciens,* and *B. fibrisolvens*. It has also been demonstrated that they have the enzymatic pool needed to continue fiber degradation (Krause et al., 2003).

In vitro Fermentation Parameters

One of the factors that aids in controlling rumen microbial activity and regulating rumen ecology is ruminal pH (Jayanegara et al., 2020). pH has been shown to influence the rumen's microbial diversity and functioning. This suggests that if the pH of the digestive system deviates from its appropriate level, microbial diversity will alter as well, which will impact the system's resistance to phytochemical effects during methanogenesis (Cerrato-Sanchez et al., 2004). Recent research revealed that ruminal pH remained constant within a range of 6.78 to 6.87 when MMALE were added. By maintaining a higher pH, enhancing NH_3-N concentration, and promoting microbial protein synthesis, plant-based BC supplementation may improve rumen efficiency (Wanapat, 2000). Moreover, MMALE addition improved NH_3-N concentration, which could be due to the role of MMALE in improving the proteolysis process. Rumen microbes can utilize nitrogen sources to produce their own protein by trapping nitrogen (Marcos et al., 2020). Similarly, Kholif et al. (2018) showed that the leaves of *Moringa oleifera* have the ability to increase NH_3-N levels and regulate ruminal pH. The characteristics of ruminal fermentation can be altered by supplementing medicinal plants to the diet (Váradyová et al., 2018).

Ruminants have a symbiotic relationship with rumen microorganisms where the animal provides nutrients and promotes optimal conditions for fermentation, while the microbes break down fiber and produce microbial protein to supply the host with protein and energy. The conversion of carbohydrates into pyruvate by fermentation in the rumen leads to the generation of metabolic hydrogen. Volatile fatty acids (VFAs) are organic hydrogen sinks that support fermentation by preserving the balance of hydrogen. Controlling the proportions of VFAs is essential for devising ways to influence rumen microbial activity (Calsamiglia et al., 2006; Lillehoj et al., 2018). In this experiment, Total VFA and propionate production were significantly increased by the supplementation with MMALE at 6% total DM substrate, which agreed with the results of Phesatcha et al. (2020), who found that the supplementation of *Mitragyna speciosa* Korth leaf in an *in vitro* did not affect butyrate production, but did increase total VFA and propionate profile. Additionally, by increasing propionate production at the expense of acetate, flavonoids improve fermentation efficiency. They also decrease the hydrogenotrophic methanogenic communities of Archaea (Seradj et al., 2014). Propionate was produced using hydrogen rather than the primary substrate for the methane synthesis pathway, which may explain why its concentration has increased (Newbold et al., 2005). Ruminant methane emissions are not only a significant source of energy loss for the animals but also a serious environmental problem. Metabolites are generated as feedstock and then further broken down and fermented by different subsequent microbial processes. Methanogenesis converts carbon dioxide and hydrogen from feed fermentation into methane, methanogenic archaea catalyze this process (Cerrilla and Martinez, 2003). The possibility for reducing the formation of methane by several anti-methanogenic compounds has already been investigated (Patra et al., 2017). However, due to their negative effects on rumen ecology, their usage is limited (Jafari et al., 2019). Therefore, the livestock industry has a huge demand for rapid and sustained methane mitigation strategies. A relatively recent and promising strategy is combining several plant extracts to reduce methane effectively and sustainably (Jayanegara et al., 2020). Methane production was reduced with MMALE supplementation, and this was also found in the current study. The direct inhibitory impact of MMALE on methanogenic archaea and protozoal groups may be the cause of the decrease in methane production. Similarly, Ahmed et al. (2021) showed that plant-based bioactive supplementation had the capacity to significantly lower methane production. As H_2 sinks, flavonoids have been proposed to inhibit ruminal methanogenesis in an indirect way (Becker et al., 2013). Other investigations have revealed that flavonoids directly inhibit methanogens (Seradj et al., 2014).

Ruminal Microbial Population

Numerous microorganisms in the rumen ecosystem ferment the feed that is consumed and produce a variety of metabolites to meet the nutritional requirements of the host (Henderson et al., 2015). There are several dietary strategies that can modify the rumen microbiota, plant-based BCs have a greater potential than antibiotics in this regard to modify the ruminal microbiota and reduce methane production through a variety of antimicrobial mechanisms including cell membrane disruption, suppression of enzymes, and prevention of bacterial group

(Reddy et al., 2020). Plants possess beneficial characteristics derived from their particular bioactive components, which are also synthesized as chemical defenses against microbial infection. Several categories can be used to group the most significant antimicrobial phytochemicals, namely essential oils, lectins/polypeptides, terpenoids, as well as phenolics/polyphenols (Windisch et al., 2008). In this study, MMALE affected cellulolytic bacteria population (*R. albus, R. flavefaciens,* and *F. succinogenes*), as the level of MMALE increased. Similarly, Chanjula et al. (2022) revealed that *Mitragyna* leaf can increase cellulolytic bacteria. Furthermore, the abundance of *B. fibrisolvens* was enhanced by MMALE supplementation. This could be due to MMALE containing BC and may affect the microbial population. With regard to microbial populations, flavonoids have a variety of biological effects that may encourage bacterial growth or change ruminal microbes, which may affect how ruminal feed is digested (Zhan et al., 2017). Accordingly, Huang et al. (2021) explained that *Paulownia* leaf which contains BCs resulted in an increase in groups of *F. succinogenes* and *B. fibrisolvens*. Importantly, MMALE was associated with decreases in Archaea, as well as in particular species of *Methanobacteriales*. An increase in propionate concentration is typically present in association with rumen methanogenesis inhibition, which was also observed in the present experiment. Petrič et al. (2020) showed that dietary substrates rich in flavonoids and phenolics may decrease methane production and result in positive rumen environment changes. The flavonoids studied *in vitro* during ruminal fermentation have shown the ability to reduce methane production during ruminal fermentation (Sinz et al., 2018). These findings agreed with Ma et al. (2017), who showed that mulberry leaf flavonoids reduce rumen methanogen populations. Current study, Matra et al. (2024) demonstrated that the addition of microencapsulated medicinal (from *Mitragyna*) leaf extracts resulted in a reduction in methanogens and methane generation.

Conclusion

The results of the present study showed that supplementing MMALE at a concentration of 6% of the total DM substrate had a substantial positive effect on the production of propionate, cellulolytic bacteria, nutrient degradability, and the decrease in methanogens and methane production during *in vitro* rumen fermentation. Therefore, MMALE, a dietary beneficial plant-based BC, has the potential to effectively modify rumen fermentation, particularly in reducing methane production. Furthermore, the results of these preliminary *in vivo* studies are seen in animal investigation.

Author's Contribution: MM conceptualization, data curation, formal analysis, methodology, writing – original draft, writing – review & editing. PT data curation, formal analysis. RP data curation, formal analysis. SP conceptualization, data curation, formal analysis, methodology. MW conceptualization, data curation, methodology, writing – original draft, writing – review & editing.

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