International Journal of Agriculture and Biosciences 2025 14(6): 1113-1121.
https://doi.org/10.47278/journal.ijab/2025.089

RESEARCH ARTICLE
eISSN: 2306-3599; pISSN: 2305-6622

In Vitro Fermentation Studies through Synergistic Effects of Antioxidant Phytonutrients Derived from Encapsulated Medical Plants

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ABSTRACT

This research was aimed to investigate the combination of microencapsulated-leaf extracts (mLEs), namely *Cannabis sativa* (m*CS*LE), *Cannabis indica* (m*CI*LE), and *Mitragyna speiosa* (m*MS*LE) under *in vitro* fermentation. The experiment was conducted to assess their efficiency on different treatments; T1: control (un-supplemented), T2: m*CI*LE+m*CS*LE, T3: m*CI*LE+m*MS*LE, T4: m*CS*LE+m*MS*LE, and T5: m*CI*LE+m*CS*LE+m*MS*LE. A completely randomized design was assigned to the supplementation of three mLEs randomly at 6% DM and various aspects of *in vitro* rumen fermentation were measured. The addition of three mLEs quadratically ($P<0.05$) mitigated the gas kinetics, specifically reducing the gas rate constant for the insoluble fraction and cumulative gas at 96 hours while linearly increasing their promoters, IVDMD, and ruminal NH_3-N. This supplementation had a positive effect to increase the concentration of propionate (C3) and total VFA concentration, whereas it decreased the VFA proportion, particularly acetate (C2), butyrate (C4), and C2:C3 ratios, and CH_4 production when compared with the control in this experiment ($P<0.05$). Additionally, all of the mLEs supplementation treatments raised the pH of the rumen, which led to more of the dominant microbial population. These included *Ruminococcus albus* followed by *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Megasphaera eldenii*, *Butyrivibro fibrisolvens*, and *Butyrivibrio proteoclasticus*. Contrarily, the mLEs addition dynamically reduced *Methanobacteriales* populations ($P<0.05$). The findings indicated that the combined of three mLEs might be a viable substitute for phytonutrient-based antioxidant supplements in animal feed additives.

Keywords: Chitosan encapsulation, Feed additives, Natural phytonutrients, Rumen manipulation, Methane emission

Article History
Article # 25-095
Received: 01-Mar-25
Revised: 09-May-25
Accepted: 13-May-25
Online First: 04-Jul-25

INTRODUCTION

Global researchers have focused on the impact of agriculture on the rumen, particularly concerning methane (CH_4) emissions from the enteric fermentation of ruminants, which play a significant role in global warming. The reduction of rumen CH_4 production relies fundamentally on practical innovations and cost-effective strategies (Wanapat et al., 2015; Vasta et al., 2019). Dietary modifications are a crucial approach to reducing ruminant CH_4 emissions by adding strategically rich phytonutrient-feeds, particularly those high in polyphenols, flavonoids, condensed tannins (CT), saponins (SP) and their antioxidative compounds, to shift fermentation pattern, type of rumen microbiomes and hence, opportunities to mitigate rumen CH_4 production (Matra et al., 2021; Phupaboon et al., 2022). The phytonutrient

Cite this Article as: Phupaboon S, Matra M, Prommachart R, Totakul P and Wanapat M, 2025. *In Vitro* fermentation studies through synergistic effects of antioxidant phytonutrients derived from encapsulated medical plants. International Journal of Agriculture and Biosciences 14(6): 1113-1121. https://doi.org/10.47278/journal.ijab/2025.089

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components affect gas production by modifying microbial activity via their capacity to coat fiber, thereby influencing its degradability and protein content (Patra and Saxena, 2009; Ku-Vera et al., 2020). Previous research, according to Ku-Vera et al. (2020), who found that *Leucaena leucocephala* peels contained essential oils in rich contents of terpenoids, tannins, saponins, and flavonoids. Results have been shown to be effective at reducing CH_4 emissions when fed at levels of up to 30–35% of ration dry matter in cattle due to their ability to impact and act on the membranes of protozoa and methanogens.

Sustainnovation has developed buzzwords like "green technology" and "bioefficiency" for animal production and environmental sustainability using the recycling of tropical fruit peel waste-products and ago-residues (Wanapat et al., 2024; Prommachart et al., 2024). The rapidly growing human population has resulted in a sharp increase in the demand for animal products, including meat, and milk, in recent years (Wanapat et al., 2015). The impact of livestock farming is still a concern regarding CH_4 emissions caused by energy loss in ruminants and how it contributes to greenhouse gas emissions into the outside environment (Vasta et al., 2019). Consequently, there are many interests in the wide selection of agro-nutrients based on medicinal plants, for example, *Cannabis indica*, *Cannabis sativa*, *Mytragyna speciosa*, and/or residues of agricultural materials, as a phytonutrient supplement with bioavailability and bioefficacy to reduce the chain of methane production resulting from animal production (Semwogerere et al., 2020; Suescun–Ospina et al., 2022; Vastolo et al., 2022). The bioefficacy of herbal plant knowledge in phytochemicals for improving ruminant health and extending meat shelf life has been examined (Brenneisen, 2007). The nutrient composition of secondary plant by-products is enhanced due to the bioavailability of dominant bioactive compounds, including terpenoids, cannabinoids, mitragynine, and polyphenols, resulting in tetrahydrocannabinol (THC) and cannabidiol (CBD) in ruminant animals (Duggan, 2021). Several studies investigating the bioavailability and bioacccessibility in tern of their plant by-products (e.g., CBD) found that they are rich and a several places to get powerful anti-inflammatory and anti-bacterial compounds, whereas CBD is a non-psychoactive phytocannabinoid (Wang et al., 2017; Phesatcha et al., 2022a; Matra et al., 2024). However, essential substances exhibit instability and susceptibility to degradation under diverse environmental conditions, including temperature, pH, and light. Additionally, their persistent flavor limits their applicability, and they may possess low solubility (Dias et al., 2017).

Microencapsulation is a cutting-edge technique in various processing techniques that allows any compound to be enclosed inside a specific material to create a tiny sphere with a diameter ranging from 1μm to several 100μm. The advantages of this technology were improved stability, enhanced bioaccessibility, imparted controlled release features and storage, as well as handing convenience, which could potentially enhance efficiency in food/feed systems (Phupaboon et al., 2022). Furthermore, this technology is recognized for its environmental benefits, utilizing various encapsulants, including plant-based proteins, carbohydrates and notably chitin and chitosan. Techniques such as spray-drying and freeze-drying are employed, making it applicable in both the food industry and as feed supplements for rumen enhancement (Phupaboon et al., 2024a). Particularly, Chitosan was selected as the encapsulant for this experiment due to its prevalent application in food-feed systems, its derivation from renewable sources, its non-toxic and non-allergenic characteristics, and its capacity for biodegradation within the digestive system (Thao et al., 2022). Several studies investigated microencapsulation of herbal plants (e.g., *Mitragyna speciosa*), essential oils (e.g., lemongrass), and fruit feel extracts (e.g., mangosteen) using chitosan as a well material in a spray-drying method (Matra et al., 2024; Phupaboon et al., 2024a). Villate et al. (2023) synthesized the chitosan-coated alginate microcapsules of *Cannabis* leaf with full-spectrum extract using the microencapsulation nozzle technique, which proposed to obtain an edible pharmaceutical-grade product, suitable for its physicochemical characterization, long-term stability in three different storage conditions, and *in vitro* gastrointestinal release. Previous research has shown that *in vitro* rumen fermentation demonstrates the efficiency of chitosan extracted from shrimp shells. This effect is likely due to a reduction in ruminal protein disappearance, an increase in C3 concentration, and a decrease CH_4 emission (Thao et al., 2022; Shah et al., 2022).

Therefore, this research hypothesized that the combination of three microencapsulated leaf extracts (mLEs) supplementation to be used as a source of essential oils and bioactive compounds including *Cannabis indica, Cannabis sativa*, and *Mitragyna speciosa* would improve *in vitro* rumen fermentation characteristics, nutrient degradability, and microbial population and mitigate CH_4 production. However, to our knowledge, there is no study investigating our hypothesis, and research has yet to be conducted on the efficacy of three herbal plant leaf extracts, based on LEs/EOs and bioactive compounds, as a strategy to enhance their interaction with the rumen microbial population and CH_4 production during an *in vitro* gas production system. Therefore, this research aimed to investigate the effect of a combination of those mLEs supplementations at 6% added to the ratio of rice straw-to-concentrate (40:60) in an *in vitro* rumen fermentation study.

MATERIALS & METHODS

Animal Ethics

This study was conducted using rumen fluid collected from dairy steers and three medical plants: *Cannabis indica* (*CI*), *Cannabis sativa* (*CS*), and *Mitragyna speiosa* (*MS*). The protocol was approved by the ethics committee of Khon Kaen University's guidelines of the Institutional Animals Care and Use Committee of Khon Kaen University and the Animal Care and Use Committee under the Institute of Animals for Scientific Purpose Development (IAD), Thailand (record no. IACUC-KKU 110/66 and U1-10937-2566).

Plant Material, Extraction and Chitosan Microencapsulation

Three leaves were allotted from different geographic localizations under Khon Kaen University and Rajamangala University of Technology Isan (Northeast of Thailand) as well as Rajamangala University of Technology Srivijaya (South of Thailand), which grew under a greenhouse and controlled management.

The phytonutrient extraction from those plant leaves was carefully dried (at 50°C for 72 hours) and ground into powder before extracting their compounds using the microwave extraction method by water extraction. Approximately, 30g of each powdered sample was used for the microwave extraction process (MIE), which required 35 minutes at 100 Watts of power and a final temperature at 60°C. Then, cellulose filter paper removed all insoluble particles, and the solution was collected for use in the next step (Phupaboon et al., 2022).

The chitosan-microencapsulation of those leaf extracts (e.g., m*CI*LE, m*CS*LE and m*MS*LE) were formulated using spray-drying techniques. For the microencapsulated powders were formulated by using 0.5L of each extracted solution combined with 0.5L of chitosan solution, as an encapsulant to retain their compounds through the spray-drying technique by a Büchi B-191 spray dryer (Buchi (Thailand) Ltd.), as outlined by Phupaboon et al. (2024a).

Experimental Design, Treatments and Chemical Analysis

The study was assigned by a completely randomized design (CRD). Total dietary substrates (rice straw-to-concentrate (R:C) ratio at 60:40) were weighed at 0.5g into bottles, and the treatments of three mLEs: m*CI*LE, m*CS*LE, and m*MS*LE were supplemented at 6% DM, which investigates a new formula and high bioaccessibility of EOs and bioactive compounds in an *in vitro* gas technique (Fig 1).

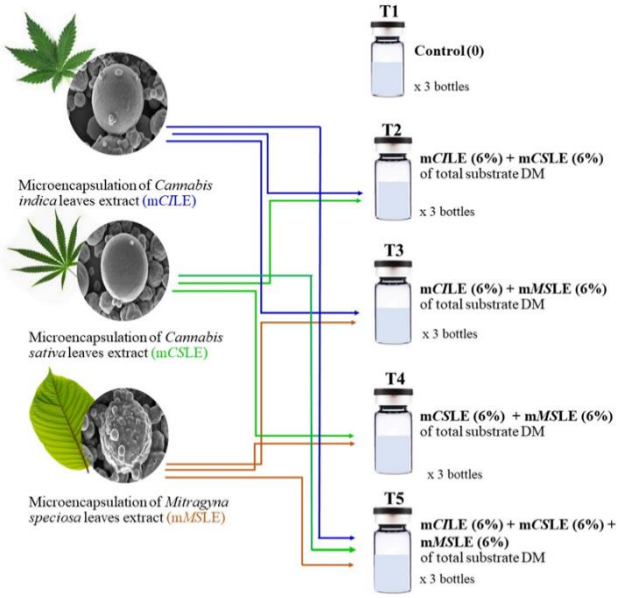

Fig. 1: The experimental design and management of the three microencapsulated supplements used *in vitro* gas fermentation experiment.

As presented in Table 1, all ingredients and mLEs underwent chemical analysis for dry matter (DM) using method 930.15, crude protein (CP) via method 990.03, ash content according to method 942.05, and ether extract following method 920.39, as outlined by Thiex et al. (2012). The NDF and ADF, were established by van Soest et al. (1991). Furthermore, each mLE supplementation was evaluated for phytonutrients according to the phytonutrient values (total polyphenolic content (TPC), total flavonoid content (TFC), DPPH assay, ABTS assay, and FRAP assay as outlined by Phupaboon et al. (2022).

Rumen Fluid and Inoculum Preparation

Two dairy steers with rumen fistulas, averaging a body-weight at 300-320kg, were utilized as donors for rumen fluid (1200mL). Animals were provided with a total mixed ration *ad libitum* twice daily, in the morning and evening, along with unrestricted access to a mineral block and clean water.

A portion of rumen fluid medium for inoculum was prepared by mixing a portion of the filtered fluid sample with artificial saliva solution in a proportion of 2:1 mL/mL, and pre-warmed in a water bath at 39°C under continuous flushing with CO_2 (Makkar et al., 1995). *In vitro* dietary substrates were pre-warmed in a water bath at 39°C prior to the addition of 40mL of mixed rumen fluid inoculum, followed by incubation at 39°C during fermentation.

***In vitro* Fermentation**

Gas production during incubation was measured at 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours (3 bottles/treatment) according to Ørskov and McDonald (1979). After fermentation, ruminal fluid was separately sampled following the inoculation times: 12, 24, and 48 hours (2 bottles/treatment) to determine the pH, ammonia nitrogen (NH_3-N), and volatile fatty acids (VFAs) as well as the rumen microbial population using the RT-PCR technique. The sampled rumen fluid was prepared into two portions. The first portion of the whole sample was collected in a sterile tube to be used for DNA extraction to identify the microbial population using the RT-PCR technique. Besides, the second portion was filtered through four layers of cheesecloth to separate insoluble fiber, and the supernatant was collected into the tube containing 1.0 M H_2SO_4 for analysis of NH_3-N content using spectrophotometry (Ahmed et al., 2021) and the concentration of the VFAs using GC equipment (GC2014; Shimadzu Co Ltd., Kyoto, Japan) (Phupaboon et al., 2024a). The CH_4 emission was calculated following the equation of CH_4 = 0.45 (acetate – 0.275 (propionate) + 0.4 (butyrate). Additionally, the *in vitro* nutrients degradability was measured by dried DM matter (van Soest et al., 1991).

Quantification of Rumen Microbial Population

The gDNA template was extracted from rumen fluid sample following the procedure of QIAamp Fast DNA Stool Mini kit (Qiagen, Germany). The designed primers: forward (Fw) and reverse (Rv) were used in this study to investigate dominant species in rumen microbiota namely *F. succinogenes, R. albus, R. flavefaciens* (Koike and Kobayashi, 2001), *M. elsdenii* (Ouwerkerk et al., 2002), *B. fibrisolvens* (Fernando et al., 2010), *B. proteoclasticus* (Paillard et al., 2007) and *Methanobacteriales* (Yu et al., 2005) through RT-PCR analysis. The RT-PCR condition was conducted by the

Table 1: Chemical composition of feeds, leaf meals, and microencapsulation of leaf extracts (mLEs) supplements

Items	C	R	*CS*LE	*CI*LE	*MS*LE	m*CS*LE	m*CI*LE	m*MS*LE
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	92.5	92.7	93.1	88.3	88.6	90.1
	----------% dry matter----------							
Organic matter (OM)	92.2	85.4	80.9	86.0	94.8	93.9	93.9	96.4
Crude protein (CP)	14.6	2.4	20.3	19.1	19.7	21.5	20.5	18.6
Neutral-detergent fiber (NDF)	20.5	78.9	43.4	45.2	48.0	70.4	70.7	72.2
Acid-detergent fiber (ADF)	8.2	52.6	26.4	26.4	19.6	22.5	23.1	21.9
Ether extract	2.8	1.2	2.0	2.1	0.5	2.2	2.5	1.1
Phytonutrition content								
TPC (mg GAE/g DM)	-	-	171.7	218.9	306.9	240.0	266.1	307.8
TFC (mg QUE/g DM)	-	-	66.2	88.6	119.2	22.6	69.8	51.3
Antioxidative values								
DPPH inhibition (%)	-	-	35.0	39.2	88.3	69.9	83.5	94.8
ABTS inhibition (%)	-	-	43.1	49.6	95.3	26.6	30.7	88.9
FRAP capacity (mg TROE/g DM)	-	-	9.4	15.1	30.4	17.2	30.9	34.4

C, concentrate; R, roughage (rice straw); *CS*LE, *Cannabis sativa* leaf extract; *CI*LE, *Cannabis indiga* leaf extract; *MS*LE, *Mitragyna speciosa* leaf extract; m*CS*LE, microencapsulated of *Cannabis sativa* leaf extract; m*CI*LE, microencapsulated of *Cannabis indica* leaf extract; m*MS*LE, microencapsulated of *Mitragyna speciosa* leaf extract; 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 10,000,000 IU; vitamin D 1,600,000 IU; vitamin E 70,000 IU; Fe 50g; Mn 40g; Zn 40g; Cu 10g; I 0.5g; Se 0.1g; Co 0.1g

CFX Connect™ Real-Time machine (Bio-Rad, Singapore) using Maxima SYBR Green qPCR Master Mix (Thermo Scientific™, USA) according to the procedure of Phupaboon et al. (2024a) and Lee et al. (2006).

Statistical Analysis

Data management and analysis were conducted utilizing a CRD platform through the GLM procedure of SAS (2013) version 9.0 (SAS Inst. Inc., NC, USA). Differences between treatment means were assessed using Tukey's test, with $P<0.05$ and $P<0.01$ indicating statistically significant differences. Responses at each mLEs level were analyzed using orthogonal polynomials.

RESULTS

Composition of Feeds and mLEs Supplements

The nutritional values of feeds as shown in Table 1, particularly rice straw and concentrate, were 89.4-90.5 for DM, 85.4-92.2 for OM, 2.4-14.6 for CP, 78.9-20.5 for NDF, and 52.6-8.2% for ADF, respectively. Subsequently, the nutrition values of *Cannabis sativa*, *Cannabis indica*, and *Mitragyna speiosa* leaf extracts (called *CS*LE, *CI*LE, and *MS*LE) ranged from 92.5–93.1, 80.9–94.8, 19.1-20.3, and 43.4–48.0% for DM, OM, CP, NDF, and ADF, separately. Also, the results for each microencapsulated leaf extract (mLE), such as m*CS*LE, m*CI*LE, and m*MS*LE, were reported to be between 88.3-90.1%, 93.9-96.4%, 18.6-21.5%, 70.4-72.2%, and 21.9-23.1%, respectively. The current results of phytonutrient content and phytochemical value ranged from 171.7–307.8 (mg GAE/g DM) for TPC, 22.6–119.2 (mg QUE/g DM) for TFC, 35.0–94.8% for DPPH inhibition, 26.6–95.3% for ABTS inhibition, and 9.4–34.4 (mg TROE/g DM) for FRAP reducing power capacity, respectively.

In vitro Gas Production Kinetics and Nutrient Degradability

Table 2 shows the cumulative gas production after 96 hours and *in vitro* DM degradability (IVDMD) of each combined mLEs treatments: T1; control; T2; m*CI*LE+m*CS*LE; T3; m*CI*LE+m*MS*LE; T4; m*CS*LE+m*MS*LE; and T5; m*CI*LE+m*CS*LE+m*MS*LE are supplemented for one level of the concentration at 6% of total DM substrate. The gas kinetics production from T1 to T4 was significantly influenced ($P>0.05$) by fraction (c): the gas production rate constant for the insoluble fraction, the potential extent of gas production (a + b), and cumulative gas production at 96 hours. Interestingly, the supplementation of T5 (m*CI*LE+m*CS*LE+m*MS*LE) was significantly affected quadratically by the gas production rate constant for the insoluble fraction (c) and cumulative gas production at 96 hours ($P>0.05$). Additionally, this experiment presents the result of IVDMD (%DM) of all treatments at different fermentation times (12, 24, and 48 hours) impacted by each supplement of mLE at 6% DM. Especially, a positive correlation in T5 was significantly increased (L; $P<0.05$) by the IVDMD at 12 and 24 hours, resulting from the fermentation time increase (Table 2).

Ruminal pH and Ammonia-nitrogen (NH_3-N) Concentrations

The results of mLE supplements at 6% of total DM substrate on *in vitro* ruminal pH were not affected ($P<0.05$), while the NH_3-N concentration was significantly influenced ($P<0.05$) by the linear score at 12 and 48 hours (Table 3). The ruminal pH values varied between 6.74 and 6.84 across all treatments during *in vitro* rumen fermentation. Likewise, supplementation of mLEs on each treatment at different

Table 2: Effect of mLEs supplements on gas production kinetics and nutrient degradability in the *in vitro* experiment

Treatment	mLEs Ratio (%DM)	Gas kinetics[1]				Cumulative gas[2] at 96 hrs	IVDMD (%DM)		
		a	b	c	a+b		12 hrs	24 hrs	48 hrs
T1	0	-2.3	102.3	0.021[a]	100.0	101.3[a]	47.2[d]	51.3	57.4[d]
T2	6:6	-1.8	97.8	0.016[b]	94.0	97.3[b]	55.8[b]	59.6	65.3[b]
T3	6:6	-1.8	96.2	0.015[b]	94.4	95.7[b]	55.2[b]	59.1	65.6[b]
T4	6:6	-1.5	96.5	0.018[b]	96.4	98.7[b]	53.1[c]	57.5	64.2[c]
T5	6:6:6	-2.5	95.3	0.011[c]	92.8	92.3[c]	56.7[a]	60.3	67.5[a]
SEM		0.52	0.68	0.03	1.12	1.53	1.56	1.68	0.96
Orthogonal polynomials									
Linear		0.17	0.25	0.14	0.35	0.16	0.03	0.62	0.03
Quadratic		0.29	0.36	0.03	0.69	0.04	0.74	0.97	0.46
Cubic		0.53	0.18	0.18	0.92	0.46	0.85	0.26	0.72

T1, control; T2, m*C*ILE+m*CS*LE; T3, m*C*ILE+m*MS*LE; T4, m*CS*LE+m*MS*LE; T5, m*C*ILE+m*CS*LE+m*MS*LE; mLEs, microencapsulation of leaf extracts; 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/hrs); (a+b), the potential extent of gas production (mL). [2]Cumulative gas at 96 hrs (mL/0.2g DM substrate). [a-c]Means with different superscripts within a column are significantly different (P<0.05); treatments are expressed as mean and values are calculated from a minimum of three replicates.

Table 3: Effect of mLEs supplementation on *in vitro* ruminal pH and ammonia-nitrogen (NH_3-N) concentration in the *in vitro* experiment

Treatment	mLEs Ratio (%DM)	pH			Ammonia nitrogen (mg/dL)		
		12 hrs	24 hrs	48 hrs	12 hrs	24 hrs	48 hrs
T1	0	6.81	6.75	6.74	14.2[a]	15.4	16.7[a]
T2	6:6	6.79	6.79	6.77	13.8[a]	16.0	18.2[b]
T3	6:6	6.81	6.80	6.76	15.8[a]	17.0	17.9[c]
T4	6:6	6.84	6.80	6.79	15.7[a]	16.8	18.6[b]
T5	6:6:6	6.82	6.79	6.75	14.7[b]	18.2	18.7[b]
SEM		0.32	0.41	0.28	0.24	0.39	0.01
Orthogonal polynomials							
Linear		0.35	0.21	0.13	0.04	0.28	0.01
Quadratic		0.46	0.53	0.24	0.78	0.80	0.41
Cubic		0.55	0.75	0.15	0.18	0.78	0.16

T1, control; T2, m*C*ILE+m*CS*LE; T3, m*C*ILE+m*MS*LE; T4, m*CS*LE+m*MS*LE; T5, m*C*ILE+m*CS*LE+m*MS*LE; mLEs, microencapsulation of leaf extracts; SEM, standard error of mean. [a-c] Means with different superscripts within a column are significantly different (P<0.05); treatments are expressed as mean and values are calculated from a minimum of three replicates.

fermentation times of 12, 24, and 48 hours demonstrated the highest ruminal NH_3-N concentrations at 18.2, 17.0, and 18.7 mg/dL when compared with T1 of 14.2, 15.4, and 16.7 mg/dL, respectively. Overall, ruminal NH_3-N concentration related to each treatment was increased when increasing the nutrient durability (IVDMD) content based on fermentation time. Especially, the T5 was steady increasing the IVDMD from 18.2 to 18.7 (mg/dL) for 12 and 48 hours, respectively (P<0.05).

Volatile Fatty Acid and Methane Production

The results of VFA profile, total VFA, and methane production at several times were affected (P<0.05) by each treatment by different mLEs supplementation (Table 4). The total VFA proportion ranging of 60.4-68.6mmol/L. Interestingly, the positive trend of T5 (m*C*ILE+m*CS*LE+m*MS*LE) supplemented at the ratio of 6:6:6% of total DM substrate increased the VFA profile, in particular propionate (C3) and total VFA concentrations, whereas lowered C2, C4 and C2:C3 ratios, along with CH_4 production, changed in range from 22 to 16% when compared with control after incubation at 48 hours (Table 4).

Dynamic Changes of Rumen Microorganisms

Seven abundant specific species, specifically *F. succinogenes*, *R. albus*, *R. flavefaciens*, *M. elsdenii*, *B. fibrisolvens*, *B. proteoclasticus*, and *methanobacterials,* were significantly different (L, Q, and C; P<0.05) with mLEs supplementations, as shown in Table 5. Interestingly, there is a dynamic change in correlation related to a slight increase in IVDMD degradability, NH_4-N values, the concentration of VFAs (C3), and total VFA that was significantly enhanced (P<0.05) on the fibrolytic and cellulolytic bacterial, namely *F. succinogenes* and *R. flavefaciens*, while lowering *R. albus* for 24 and 48 hours. In addition, acidobacteria, *M. elsdenii*, and hydrogenation bacteria consisting of *B. fibrisolvens* and *B. proteoclasticus* were affected for 48 hours after *in vitro* fermentation. Mostly, *R. albus* was the dominant species (10^8 copies/mL), followed by *R. flavefaciens* and *M. elsdenii* (10^7 copies/mL), along with *F. succinogenes, B. fibrisolvens*, and *B. proteoclasticus* (10^7 copies/mL), those species that convert the nutrient complex changes for energy and/or essential amino acid sources in their cell growth. Additionally, our result confirms that the population of methanobacteria as methane producers, specifically *Methanobacteriales* (10^4 copies/mL) consisting of references: *Methanobacterium bryantii* (DSM 863), *Methanabrevibacter arboriphilicus* (DSM 1536), Methanosphara stadmanae (DSM 3091), and *Methanobacterium themoautotrophicum* (DSM 1053) was significantly decreased (P<0.05) among copies at 12 and 48 hours. This result is consistent with methane production showing decreased production with the duration of herbal phytochemical components release within the microencapsulated vesicles. This phenomenon can be attributed to the concentration of phytonutrient compounds (e.g., TPC, TFC, and/or antioxidant capacity) retained by mLEs, which are rapidly released during the initial stages of *in vitro* fermentation. The reduction of CH_4 production is therefore inadequate.

Table 4: Effect of mLEs supplementation on *in vitro* volatile fatty acids (VFA), total VFA, and methane (CH_4) productions in the *in vitro* experiment

Treatment	mLEs Ratio (%DM)	VFA (mol/100 ml)			C2:C3	Total VFA (mmol/L)	Methane production (%)		
		C2	C3	C4			12 hrs	24 hrs	48 hrs
1	0	62.8^{b}	25.9^{c}	11.3	2.47^{a}	60.4^{d}	17.7^{a}	21.9^{a}	25.5^{a}
2	6:6	63.4^{a}	25.6^{c}	11.0	2.43^{a}	62.0^{c}	17.1^{a}	20.4^{b}	23.0^{b}
3	6:6	63.5^{a}	26.3^{c}	10.2	2.41^{a}	62.6^{c}	17.3^{a}	20.6^{b}	23.2^{b}
4	6:6	62.7^{b}	27.2^{b}	10.0	2.30^{b}	66.1^{b}	16.7^{b}	20.0^{b}	22.6^{bc}
5	6:6:6	60.2^{c}	28.6^{a}	10.2	2.21^{c}	68.6^{a}	14.7^{b}	19.0^{c}	20.6^{c}
SEM		0.52	0.63	0.74	0.05	1.35	0.64	0.78	0.85
Orthogonal polynomials									
Linear		0.37	0.03	0.46	0.15	0.57	0.03	0.01	0.57
Quadratic		0.24	0.86	0.53	0.04	0.69	0.55	0.35	0.78
Cubic		0.04	0.82	0.34	0.26	0.04	0.76	0.17	0.04

T1, control; T2, m*CI*LE+m*CS*LE; T3, m*CI*LE+m*MS*LE; T4, m*CS*LE+m*MS*LE; T5, m*CI*LE+m*CS*LE+m*MS*LE; mLEs, microencapsulation of leaf extracts; VFA, volatile fatty acids; C2, acetate; C3, propionate; C4, butyrate; C2:C3, acetate to propionate ratio; SEM, standard error of mean. $^{a-d}$Means with different superscripts within a column are significantly different (P<0.05); treatments are expressed as mean and values are calculated from a minimum of three replicates.

Table 5: Effect of mLEs supplements on rumen microbial population in the *in vitro* experiment

Species	Time (hrs)	Treatment					SEM	Orthogonal polynomials		
		T1	T2	T3	T4	T5		L	Q	C
F. succinogenes, (10^7 copies/mL)	12	0.2^{a}	0.1^{b}	0.07^{c}	0.09^{c}	0.1^{b}	0.71	0.01	0.16	0.58
	24	0.1	0.1	0.1	0.1	0.2	0.71	0.68	0.78	0.85
	48	0.3^{a}	0.1^{b}	0.1^{b}	0.2^{c}	0.2^{c}	0.11	0.37	0.01	0.33
R. albus, (10^8 copies/mL)	12	0.7^{a}	0.1^{b}	0.1^{b}	0.2^{c}	0.08^{b}	0.13	0.01	<0.01	0.26
	24	0.6	0.3	0.2	0.5	0.1	0.23	0.65	0.12	0.99
	48	1.8	1.9	2.1	2.1	0.8	0.58	0.60	0.86	0.86
R. flavefaciens, (10^7 copies/mL)	12	1.4	0.6	0.6	1.1	0.5	0.40	0.45	0.06	0.85
	24	2.9^{a}	0.9^{b}	0.7^{b}	0.7^{b}	0.5^{b}	0.39	<0.01	<0.01	0.15
	48	9.5^{a}	2.1^{b}	2.2^{b}	1.9^{b}	1.4^{b}	1.04	<0.01	<0.01	0.04
M. elsdenii, (10^7 copies/mL)	12	1.2	0.9	0.8	0.8	0.7	0.35	0.25	0.39	0.90
	24	1.5	1.1	0.7	0.7	0.7	0.41	0.06	0.46	0.68
	48	2.7^{a}	2.7^{a}	1.6^{b}	1.3^{c}	1.3^{c}	0.48	0.01	0.65	0.27
B. fibrisolvens, (10^7 copies/mL)	12	1.9^{a}	0.8^{b}	0.7^{b}	0.5^{b}	0.8^{b}	0.32	<0.01	0.09	0.44
	24	0.8	1.1	0.5	0.5	0.5	0.22	0.08	0.34	0.06
	48	1.5^{a}	2.7^{b}	2.6^{c}	1.8^{d}	1.5^{a}	0.39	0.55	<0.01	0.54
B. proteoclasticus, (10^7 copies/mL)	12	4.1	5.0	4.9	4.8	4.9	0.99	0.53	0.48	0.72
	24	4.2	5.0	4.0	4.8	4.5	0.73	0.68	0.99	0.17
	48	3.5^{a}	5.5^{b}	6.3^{c}	4.7^{d}	4.4^{d}	0.24	<0.01	<0.01	0.15
Methanobacteriales, (10^4 copies/mL)	12	5.0^{d}	14.4^{a}	11.4^{b}	8.5^{c}	10.1^{c}	0.34	0.47	0.03	0.26
	24	4.6	4.6	2.6	3.9	5.2	0.92	0.17	0.30	0.10
	48	2.4^{b}	3.6^{a}	3.7^{a}	3.4^{a}	0.6^{c}	0.16	0.01	0.48	0.88

T1, control; T2, m*CI*LE+m*CS*LE; T3, m*CI*LE+m*MS*LE; T4, m*CS*LE+m*MS*LE; T5, m*CI*LE+m*CS*LE+m*MS*LE; mLEs, microencapsulation of leaf extracts; SEM, standard error of mean; L, linear; Q, quadratic; C, cubic. $^{a-c}$Means with different superscripts within a column are significantly different (P<0.05); treatments are expressed as mean and values are calculated from a minimum of three replicates.

DISCUSSION

The hypothesis of this research was to evaluate the effect of microencapsulated herbal plant extract-based EOs and phytonutrients formulated with 1% of chitosan, which was used in this experiment. It revealed notable findings that a biomaterial, chitosan, was used as a coating to formulate microcapsules through the microencapsulation technique. The current findings have greatly enhanced our understanding of chitosan microencapsulation, which increases the content of OM, CP, and the remaining phytonutrient compounds. Chitosan is formed as a wall material in the particle encapsulation process. When chitosan is burned at a high temperature or even dissolved in concentrated acid. It decomposes into free nitrogen and becomes organic matter. In addition, it is also able to release the retained bioactive substances, thereby resulting in higher contents after encapsulation than samples without encapsulation by microencapsulation technology (Phupaboon et al., 2022).

In particular, as a results from Table 1, the *MS*LE and m*MS*LE showed higher phytonutrient contents in terms of antioxidant values than the *CS*LE, *CI*LE, m*CS*LE, and m*CI*LE. The findings of the current study are consistent with those reported by Phupaboon et al. (2022) and Goh et al. (2021), *Cannabis* and/or *Mitragyna* species leaf extracts contained the majority of phytonutrient compounds: total polyphenol content (287.2-407.8mg GAE/g), total flavonoid content (119-194.0mg QUE/g), and antioxidant capacity in terms of DPPH radical scavenging inhibition (39.2-46.8%). Furthermore, numerous studies have reported their estimated solubility in different conditions, specifying the concentration of other phytogenic compounds from either *Cannabis* or *Mitragyna* species, such as condensed tannins (8.28–14.6%), saponin (5.21–12.1%), cannabidiol (2.8–16.5mg/g), tetrahydrocannabinol (0.011–1.250mg/g), mitragynine (4.14–8.2%), paynantheine (0.59%) and speciogynine (8.28%), which applied for the bioefficacy and bioaccessibility in different fields of medicinal, functional food, and ruminant nutrition (Semwogerere et al., 2020; Phesatcha et al., 2022a; Chanjila et al., 2022).

The most interesting thing about the data is that the addition of T5 (m*CI*LE+m*CS*LE+m*MS*LE) decreased the number of gases produced and the concentration of NH_3-N while increasing IVDMD values. Previous research has examined the systematic metabolization of *C. indica* and/or *C. sativa* from leaves, cake, and seed oil extract mixed with animal feed. The studies noted significantly reduced total gas and CH_4 productions, alongside substantial effects on feed degradability and volatile fatty

acid patterns (Kleinhenz et al., 2020; Jensen et al., 2022). Phesatcha et al. (2022a) found that adding *M. speciosa* leaf powder (MSLP) at levels of 0, 1, 2, 3, 4, 5, 6, and 7% of the total dry matter (DM) substrate increased when the ratio of R:C was 40:60, which also influenced the insoluble fraction. According to previous research of Phesatcha et al. (2022b), who reported the effect of MSLP at concentrations from 0 to 7% of total DM substrate added into the R:C ratio (at 60:40 and 40:60), there was no significant interaction on the %DM of IVDMD and IVOMD for incubation after 12 and 24 hours. Wang et al. (2017) established the lipophilic antioxidants from unconventional oilseeds: hemp (*C. sativa*), safflower seeds, and coconut oil, were affected by IVOMD more than IVDMD of untreated, with up to 11% related to methane reduction. Similarly, Gerlach et al. (2018) discovered the highest concentration of phytonutrient values: CT and SP can inhibit the population of ruminal fibrolytic bacteria that are important for rumen digestibility. Moreover, tannins can combine with proteins and carbohydrates through interactions with either hydrogen atoms or hydrophobic molecules, and homo- or heterofermentative microorganisms decrease their susceptibility to fermentation (Vasta et al., 2019; Jayanegara et al., 2020).

The results align with prior research of Paula et al. (2020) indicated that the neutral pH at 6.6 encompasses slightly acidic conditions (6.1–6.5) in feed with high roughage attention, which is essential for sustaining microbial growth, especially cellulolytic bacteria. Furthermore, ruminal NH_3-N profiles ranged from 15.8-30.0mg/dL incubated with extracted-chitosan and -shrimp shell at R:C ratios 60:40 and 40:60 (Thao et al., 2022). An additional significant finding indicated that the supplementation of dragon fruit peel powder (DFPP) as a phytonutrient substrate resulted in an increase in mean NH_3-N concentration at 14.3-20mg/dL, which is conducive to nutrient degradability and microbial protein synthesis (Matra et al., 2021). The results contrast with certain published studies, which indicated that MSLP supplementation in either powder or pellet forms for Thai native beef cattle and/or goats, utilized as a phytonutrient compound, led to reduced ruminal NH_3-N concentrations, likely attributable to CT and SP in DM. The protein degradation was safeguarded, leading to the formation of protein combined with CT structure. In the presence of inhibitors, this complex reduced protein degradability and adversely affected the rumen microbiota by inhibiting NH_3-N synthesis (Phesatcha et al., 2022a; 2022b; Chanjila et al., 2022).

Additionally, these results were similar to those findings, who reported that the addition of *M. speciosa* leaf powder and *C. sativa* leaf extract type *Finola* F25 into feed significantly increased the total VFA and C_3 concentration ($P<0.05$) and decreased the proportion of the C_2:C_3 ratio (Chanjila et al., 2022; Jensen et al., 2022). The current findings align with existing research, indicating that certain concentrates contain highly biodegradable carbohydrates, such as cassava chips. In order to enhance total VFA and C_2, C_3, and C_4 in their cells, the rumen bacteria metabolized soluble carbon sources with a high-starch diet, resulting in fermentation towards C3 (Chanjila et al., 2022; Phesatcha et al., 2022b).

The current results similar to those findings found that MSLP supplementation significantly increased ($P<0.05$) on total bacteria, e.g., *F. succinogenes*, and *R. albus* (Phesatcha et al., 2022a). Contrary to the earlier findings of Wanapat and Cherdthong (2009), which indicated that *F. succinogenes* were dominant types in the digesta and rumen fluid of swamp buffalo at concentrations ranging from 10^6 to 10^9 copies/mL, followed by *R. flavefaciens* (10^5 to 10^6 copies/mL) and *R. albus* (10^4 to 10^6 copies/mL), respectively. Hung et al. (2013) and Naumann et al. (2017) hypothesized that reductions in protozoa populations could influence methanogen populations, including *Methanobrevibacter*. Methanogens engage in biological interactions with protozoa and supply hydrogen as a precursor for CH_4 synthesis. This also accords with our earlier observations, which showed that a possible explanation for this was that after MSLP supplementation, the rumen production of protozoa, methanogen-archaea, and CH_4 may have decreased as a result of phytogenic substances similar to CT and SP, as well as cannabinoids or therapies found in Cannabis seed oils (Pojić et al., 2014; Wang et al., 2017; Kleinhenz et al., 2020).

The results of this study match those of Matra et al. (2024), who discovered that adding microencapsulated *Mitragyna* leaves extracts significantly increased the IVDMD between 12 to 48 hours, and also raised the NH_3-N concentration. Supplementation with 6% of DM substrate yielded the maximum output of C3 proportion and total VFA by mMLE, while simultaneously reducing methane emissions over 12-48 hours. Additionally, mMLE feeding resulted in an increase in the population of cellulolytic bacteria and *B. fibrisolvens*, while the population of *Methanobacteriales* decreased. Recently, *in vitro* studies have shown that the efficiency of the microencapsulated phytogenic-based essential oils or plant secondary components from various plants (e.g., hemp, *Wolffia globosa*, lemongrass mixed with dragon fruit peels) can enhance and modulate ruminal *in vitro* fermentation characteristics, end-product formation, and microbial dynamics, as well as ruminal mitigation CH_4 emission (Phupaboon et al., 2024b; Muslykhah et al., 2024; Suriyapha et al., 2025).

Conclusion

Our results showed that the combination of microencapsulation leaf extracts (mLEs) of m*CI*LE and m*CS*LE, or m*MS*LE supplements, is rich in EOs and phytonutrient compounds, especially polyphenolic, flavonoid, and antioxidant capacity. Particularly, the combination of three microencapsulated herbal plant leaf extracts in T5 (m*CI*LE+m*CS*LE+m*MS*LE) supplemented in an *in vitro* rumen fermentation significantly influenced propionate production in the rumen fermentation, the dynamics of rumen microbial diversity, gas production kinetics, and methane mitigation. Moreover, future research should be done to facilitate those mLEs required for long-term efficacy and to elaborate on the potential interactions between substances in *in vivo* experiments.

DECLARATIONS

Funding: The research was conducted from research funds granted by the Khon Kaen University vide the Fundamental Fund (FF) (grant no. 65A103000130) funding from the National Science Research and Innovation Fund (NSRF) of Thailand.

Acknowledgement: The authors express gratitude to the Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand, for their support of laboratory facilities and assistance. We extend our gratitude to Dr. Peter Rowllinson for his contributions to the English language editing of the manuscript. Acknowledgment is given to the Cannabis Research Institute at Khon Kaen University and Rajamangala University of Technology Isan (RMUTI), Thailand, for their provision of plant samples and support in research collaborations.

Conflict of Interest: The authors declare no conflict of interest with any individual, company or organization.

Data Availability: The datasets generated and analyzed during this study are available within the article

Ethics Statement: All experimental animals allowed the Faculty of Animal Science farm at Khon Kaen University, Khon Kaen, Thailand. Additionally, all procedures were conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Khon Kaen University and the Institute of Animal Science for Scientific Purposes Development (IAD) in Thailand. The pertinent records for these procedures include IACUC-KKU-110/66 and U1-10937-2566.

Author's Contribution: SP and MW conceived and designed the experiment. SP, MM, PT and RP performed the study and conducted lab analyses. SP supervised, MM and PT coordinated the experiments and RP provided feed formula. SP performed statistical analyses of experimental data and prepared the manuscript format. SP and MW prepared the draft of the manuscript. All authors critically revised the manuscript and approved the final version.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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