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In vitro fermentation characteristics, microbial changes and gas production of microencapsulated phytonutrient pellets at varying dietary crude protein levels

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The objective of this study was to investigate the influence of crude protein (CP) levels combined with the supplementation of a microencapsulated phytonutrients pellet made from a mixture of lemongrass and dragon fruit peel (MiEn-LEDRAGON) on gas production, degradability, fermentation characteristics, and microbial diversity using the in vitro gas technique. A 4 × 2 factorial arrangement in a completely randomized design (CRD) was used in this study, with four levels of CP in the concentrate diet (10, 12, 14, and 16% dry matter; DM) combined with two levels of MiEn-LEDRAGON supplementation (0 and 3% in the total DM substrate). The results of this study demonstrated that there were no interaction effects between CP levels and MiEn-LEDRAGON supplementation on gas production, degradability, fermentation characteristics, end-product formation, or microbial dynamics ($p > 0.05$). Additionally, increasing CP levels in the concentrate diet had no effect on cumulative gas production, gas kinetics, in vitro degradability, volatile fatty acids (VFA), or methane (CH_4) production ($p > 0.05$), but it did enhance in vitro pH and ammonia-nitrogen (NH_3-N), as well as increase the number of *Fibrobacter succinogenes* at 24 h (h) of incubation time ($p < 0.05$). Meanwhile, the study revealed higher cumulative gas production, degradability, NH_3-N, pH values, total VFA at 24 h of incubation, proportions of propionate (C3) at 12 and 24 h of incubation, and butyrate (C4) at 12 h of incubation, as well as increased numbers of *F. succinogenes*, *Butyrivibrio fibrisolvens*, and *Butyrivibrio proteoclasticus* at 12 h of incubation when supplemented with 3% MiEn-LEDRAGON in the total DM substrate ($p < 0.05$). It also decreased the proportion of acetate (C2), CH_4 production, and the populations of methanogens (*Methanobacteriales*) and *Ruminococcus* species (*Ruminococcus albus* and *Ruminococcus flavefaciens*) ($p < 0.05$). In summary, this study found that increasing CP levels in the concentrate diet did not negatively affect gas production, fermentation characteristics, end-product formation, or microbial dynamics. Moreover, MiEn-LEDRAGON supplementation could serve as an effective rumen-enhancing feed additive rich in phytonutrients for ruminants while also mitigating ruminal CH_4 production.

Keywords Plant secondary bioactive, Tropical plants, Fruit by-product, Rumen microbiome, Methane production

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Ruminants play a crucial role in livestock production by efficiently converting agricultural by-products and fibrous biomass into protein sources for food products like meat and milk[1]. However, their production is associated with significant enteric methane (CH_4) emissions, contributing to 30% of atmospheric methane and 6% of global anthropogenic greenhouse gas emissions[2–4]. From an animal performance perspective, ruminal CH_4 production results in a loss of gross energy intake[2]. Therefore, sustainable strategies to mitigate CH_4 emissions are necessary to improve forage conversion efficiency while safeguarding the negative impact on the environment and ensuring animal health[2,4,5].

Currently, utilizing local herb and fruit peel by-products from commercial fruit canning facilities or farms as feed additives, is an interesting alternative for ruminant feed and may help reduce ruminal CH_4 emissions[5,6]. These fruit peels and herbs are rich in phytonutrient active compounds, including phenolic compounds, saponins, tannins, flavonoids and antioxidants—plant-derived nutrients that have the potential to improve animal health, enhance ruminal fermentation, boost ruminant productivity, and lower ruminal CH_4 emissions[6–9]. These plant-based bioactive compounds can decrease methane production in the rumen by reducing the population of microbial CH_4 producers (methanogens)[8,10]. Several studies[6,7,9,11–14] have shown that tropical herb and fruit peels, as well as mixed fruit peel powders or pellets, can serve as effective feed additives or supplements for ruminant nutrition.

Nano and microencapsulation techniques are applied to improve the stability, enhance bioavailability, provide specific controlled release characteristics, and facilitate easier handling and storage of target substances that act as active core components encased in polymer barriers or carrier systems[15]. These methods can safeguard the core active ingredients from harmful environmental factors, such as elevated temperatures, light exposure, pH shifts, and oxygen, which could otherwise negatively impact the product's chemical and physical characteristics[16,17]. Recently, Suriyapha et al.[5] successfully developed microencapsulation with cricket protein wall carrier to retain high-quality phytonutrient active compounds extracted from the blending of lemongrass and dragon fruit peel (MiEn-LEDRAGON), which proved effective in conserving high levels of bioactive components. The previous finding[5] indicated that supplementing with MiEn-LEDRAGON (3% in the total DM substrate) increased degradability, fermentation end-products and cumulative gas production, while also enhancing microbial diversity. Additionally, it reduced both methanogen populations and methane output, indicating its potential as a phytonutrient-rich feed additive for ruminants[5].

However, the fundamental assessment of the impact of phytonutrients from MiEn-LEDRAGON, in combination with varying CP levels in concentrate diet on in vitro degradability, fermentation characteristics, gas production, and CH_4 production has not yet been explored. This study hypothesizes that phytonutrients from MiEn-LEDRAGON, when combined with different CP levels in concentrate, could improve ruminal protein metabolism efficiency, enhance ruminal fermentation, and reduce CH_4 emissions. Therefore, the objective of the study was to investigate the influence of CP levels combined with MiEn-LEDRAGON supplementation on degradability, gas production, fermentation characteristics, and microbial changes using the in vitro gas technique.

Materials and methods

The Animal Ethics Committee of Khon Kaen University approved the use of all experimental animal donors and all methodologies employed in this study (approval number: IACUC-KKU-110/66). Moreover, this study was performed in accordance with relevant guidelines and regulations, and all methods were reported in accordance with ARRIVE guidelines.

Microencapsulation of phytonutrients product pellet preparation

The preparation of MiEn-LEDRAGON followed the procedure outlined by Suriyapha et al.[5]. In brief, the phytonutrient pellet known as LEDRAGON is composed of a blend of lemongrass and dragon fruit peel. The formulation includes 50% on a dry matter basis (DM) of lemongrass powder, 45% DM of dragon fruit peel powder, 3% DM of molasses, and 2% DM of cassava powder. These components were mixed with water to achieve a moisture content of approximately 65%, then processed through a pelletizing machine to produce LEDRAGON pellets. Afterward, they were dried in a hot air oven at 60 °C for 48 h to reduce the moisture content to below 10%. The pellets were then kept in a sealed container for the duration of the experiment. To extract the secondary bioactive compounds, the LEDRAGON pellets were ground into powder and subjected to microwave extraction. Specifically, 5 g of the pellet powder were extracted with 100 mL of deionized water at 100 watts for 35 min. The resulting extract was microencapsulated using ionic gelation combined with surfactants, incorporating a phosphate buffer solution with cricket protein (pH 5.5, 10% w/v). Subsequently, the mixture was stirred with surfactants in a 1:1 ratio at room temperature overnight. A spray dryer (Büchi B-191 mini spray dryer, Büchi Labortechnik AG, Flawil, Switzerland) was used for microencapsulation powder. The final product (MiEn-LEDRAGON) was collected, securely sealed, and stored at -20 °C until it was required for analysis and the in vitro study.

Experimental design and dietary treatment analysis

This study was conducted with two replications run using a 4 × 2 factorial arrangement in CRD, which was used to organize the experimental treatments with three replications per treatment. Factor A consisted of four levels of CP in the concentrate diet (10, 12, 14, and 16% DM), while Factor B included two levels of MiEn-LEDRAGON supplementation (0 and 3% DM in the diet). All experimental treatments had a roughage-to-concentrate (R: C) ratio of 60:40, with rice straw as the roughage base. The experimental diets were dried in a hot air oven at 60°C for 48 h, then ground through a 1-mm sieve using a Cyclotech Mill (Tecator, Sweden) for the purpose of analyzing their chemical composition. These diets were then utilized in the in vitro trial. The standard procedures of AOAC[18] were evaluated for dry matter (DM; ID 967.03) and ash (ID 492.05) for all samples. Neutral detergent

fiber (NDF) and acid detergent fiber (ADF) were assessed according to the procedures by Van Soest et al.[19]. An automated nitrogen analyzer (Leco FP828, LECO Corp., Saint Joseph, MI, USA) was used to determine the nitrogen (N) and CP contents. The total flavonoid, phenolic, and antioxidant contents were assessed following the procedure described and modified by Phupaboon et al.[15]. The Folin-Ciocalteu reagent was used to measure and calculate the total phenolic content[20], while colorimetric changes using a 10% aluminum chloride solution were employed to determine the total flavonoid content[21]. Antioxidative capacity was evaluated using three methods: the DPPH radical scavenging assay (2, 2-diphenyl-1-picrylhydrazyl)[22], the ABTS radical scavenging assay (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid))[23], and the ferric reducing antioxidant power (FRAP) method[24]. The compositions and all ingredients of the experimental diets are listed in Table 1.

Experimental animal donors and ruminal inoculum preparation

Four 6-year-old Thai-Holstein crossbred dry cows from the Dairy Unit, Department of Animal Science, Faculty of Agriculture, Khon Kaen University, with an average weight of 480 ± 10.0 kg, were used as ruminal fluid donors. The donor cows were fed total mixed ration (TMR) that was with rice straw-based *ad libitum* (65% DM of total digestible nutrient (TDN) and 12% DM of CP) twice a day, at 7:30 am and 3:30 pm. Each cow was housed individually with unrestricted access to mineral blocks and clean water. The cows were fed this diet for 21 days to allow ruminal ecology adaptation before rumen fluid collection. Rumen fluid was collected via oral suction using a vacuum pump, extracting 325 mL from each donor before the morning feeding for each experimental run. The rumen fluid was first collected via an oral suction tube into an Erlenmeyer flask, then strained through four sheets of cloth into preheated thermos containers. Finally, the ruminal fluid was delivered to the laboratory. Ruminal inoculant mixture was prepared following the procedure described by Makkar et al.[25]. A total of 1300 mL of donor cow mixed rumen fluid was combined with 4434.24 mL of ruminal-buffered mixed medium (2156.82 mL of distilled water, 1437.88 mL of buffer solution, 718.93 mL of macro mineral solution, 0.46 mL of micro mineral solution, 1.97 mL of resazurin solution, and 118.18 mL of reduction solution) at a pH of $6.5 \leq pH < 7$, under CO_2 flushing to maintain anaerobic conditions and swirled at 39 °C[25]. Then, 40 mL of the ruminal inoculant mixture was added to each experimental bottle containing 500 mg of the feed sample[25] (8 treatments + 1 blank with three replicates, resulting in a total of 27 bottles for each parameter: gas production, in vitro degradability at 12 and 24 h, and fermentation characteristics at 12 and 24 h, for a total of 135 bottles per run). The bottles were then incubated in a shaking water bath (BS-31, Jeio Tech Lab Companion, Korea) with

Item	CP10	CP12	CP14	CP16	Rice straw	Dragon fruit peel	Lemongrass	MiEn-LEDRAGON
Ingredient, % of dry matter								
Cassava chip	57.0	56.3	55.6	54.9	–	–	–	–
Rice bran	12.0	12.0	12.0	12.0	–	–	–	–
Palm kernel meal	15.0	15.0	15.0	15.0	–	–	–	–
Soybean meal	12.0	12.0	12.0	12.0	–	–	–	–
Urea	0.0	0.7	1.4	2.1	–	–	–	–
Molasses	1.5	1.5	1.5	1.5				
Sulphur	0.5	0.5	0.5	0.5	–	–	–	–
Salt	1.0	1.0	1.0	1.0	–	–	–	–
Mineral premix[a]	1.0	1.0	1.0	1.0	–	–	–	–
Chemical compositions								
Dry matter (DM), %	90.9	90.8	91.0	90.8	90.2	93.4	69.1	92.1
Organic matter, % DM	92.4	92.2	92.0	92.0	85.4	96.2	90.0	98.9
Crude protein, % DM	10.2	12.1	14.2	16.1	2.4	5.3	4.0	26.5
Neutral detergent fiber, % DM	21.0	20.9	20.4	20.0	78.9	37.0	66.4	60.3
Acid detergent fiber, % DM	13.0	12.8	12.6	12.4	52.6	28.8	40.4	20.2
Total phenolic contents, mg GAE/g DM	–	–	–	–	–	852	386	1,253
Total flavonoid contents, mg QUE/g DM	–	–	–	–	–	62	8	263
DPPH[a], mg TROE/g DM	–	–	–	–	–	2,982,498	1,963,612	2,430,277
ABTS[c], mg TROE/g DM	–	–	–	–	–	1,472,499	1,664,722.1	1,756,943
FRAP[d], mg TROE/g DM	–	–	–	–	–	23,559	7,493	13,193

Table 1. Ingredients and chemical composition of experimental diets. [a]Minerals and vitamins (each kg contains): vitamin A: 10,000,000 IU, vitamin E: 70,000 IU, vitamin D: 1,600,000 IU, Fe: 50 g, Zn: 40 g, Mn: 40 g, Co: 0.1 g, Cu: 10 g, Se: 0.1 g, I: 0.5 g; [b]DPPH = 2,2-diphenyl-1-picrylhydrazyl; [c]ABTS = 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); [d]FRAP = ferric reducing ability power; MiEn-LEDRAGON = microencapsulation of phytonutrients pellet product from lemongrass mixed dragon fruit peel; CP10 = concentrate diet containing 10% DM of crude protein; CP12 = concentrate diet containing 12% DM of crude protein; CP14 = concentrate diet containing 14% DM of crude protein; CP16 = concentrate diet containing 16% DM of crude protein.

reciprocating motion at a rotation speed of 20 rpm at 39 °C, which was modified from the method of Arowolo et al.[26].

In vitro gas production and kinetics of gas

Gas production was measured using a glass syringe injection at sample bottles to determine the gas volume at each time point, with recordings taken immediately after incubation at the following time points: 0, 0.5, 1, 2, 4, 6, 8, 12, 18, 24, 48, 72, and 96 h. The data were fitted to the model proposed by Ørskov and McDonald[27] using Fitcuve (software version 6, International Feed Resources Unit, MLURI, Aberdeen, UK), based on the following equation:

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

where: y represents the volume of gas generated at time t, a signifies the soluble fraction of gas production, b designates the insoluble fraction of gas production, c is the rate constant for gas production from the insoluble fraction (b), and t indicates the incubation duration.

The model for cumulative gas production followed the description by Pitt et al.[28] and is based on the following equation:

$$V(t) = VF \times F(t)$$

where: V(t) represents the cumulative gas produced (mL) from a given mass of substrate incubated for time, VF is the final gas volume (mL) from the complete digestion of the substrate, F(t) is the gas production function, and t is the incubation time (h).

In vitro degradability, fermentation characteristics and microbial DNA analysis

At 12 and 24 h of incubation, CH_4 was collected from the headspace of the in vitro degradability bottles by injecting 10 mL of gas into vials, following the procedure described by Kaewpila et al.[29], and analyzed using a gas chromatography system (GC; Nexis GC-2030, Shimadzu Corp., Kyoto, Japan) with a capillary column (SH-Rt-Q-BOND 30 m, 0.53 mm, 20 µm, Shimadzu Corp., Kyoto, Japan) and high-purity methane as the reference standard gas. At these time points, in vitro degradability, including in vitro dry matter degradability (IVDMD) and in vitro organic matter degradability (IVOMD), was assessed using the methods of Tilley and Terry[30]. Additionally, the fermentation bottles were opened at 12 and 24 h of incubation for in vitro sample collection and subsequent fermentation characteristics analysis. At these time points, in vitro pH was immediately measured using a digital pH meter (Brenchtop pH 700, EUTECH, Singapore). The in vitro inoculant fluid was collected at specific time points and divided into two parts: one part (25 mL) was mixed with 5 mL of 1 M H_2SO_4 and stored at -20 °C for NH_3-N and volatile fatty acids (VFA) analysis. The in vitro NH_3-N concentration was analyzed using the method described by Fawcett and Scott[31] with a UV/VIS spectrophotometer (PG Instruments Ltd., London, UK). The concentrations of VFAs, specifically acetate (C2), propionate (C3), and butyrate (C4), were analyzed using high-performance liquid chromatography (HPLC; Shimadzu LC-20 A, Shimadzu Corp., Kyoto, Japan) following the procedure of Samuel et al.[32]. Phosphoric acid (25 mM) was used as the mobile phase in an Inertsil ODS-3 C18 column (250 mm × 4.6 mm i.d., 5 µm, Shimadzu LC-20 A, Shimadzu Corp., Kyoto, Japan) with a flow rate of 1 mL/min, an injection volume of 20 µL, and UV detection at 210 nm. An additional 10 mL was placed in a new sample bottle and stored at -20 °C for the extraction of microbial DNA. Microbial DNA was isolated from the in vitro inoculant fluid and extracted with the technique established by Koike and Kobayashi[33], and purified with the QIAgen DNA Mini Stool Kit (QIAGEN, Valencia, CA, USA). Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), DNA templates, forward and reverse primers, and genomic DNA that had been obtained were all utilized in the process of real-time quantitative PCR. Specific primers were employed to measure the populations of *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus*[34], *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*[33], *Megasphaera elsdenii*[35], and *Methanobacteriales*[36]. Real-time PCR amplification and detection were performed using the Chromo 4™ system from Bio-Rad (Hercules, CA, USA) according to the DNA analysis guidelines of Koike and Kobayashi[33].

Statistical analysis

All experimental data were statistically analyzed as a 4 × 2 factorial arrangement in CRD using SAS's GLM method (Version 9.4; SAS Institute Inc., Cary, NC, U.S.A)[37]. The following model was used to analyze the data:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$$

where: Y_{ij} represents observation values; µ is overall mean; α_i represents effect of main factor A (the CP levels at 10, 12, 14, 16% DM in the concentrate diet when $i = 1$ to 4); β_j is effect of main factor B (the MiEn-LEDRAGON supplementation at 0 and 3% in the total DM substrate when $j = 1$ to 2); $\alpha\beta_{ij}$ designates interaction of A and B at ij; ε_{ij} indicates error term. The mean values were compared at $p < 0.05$ using Duncan's new multiple range tests, which is considered a statistically significant difference.

Results

In vitro gas production and kinetics of gas

The influence of CP levels combined with MiEn-LEDRAGON supplementation on gas production and kinetics of gas at 96 h after incubation is shown in Table 2. There was no interaction effect between CP levels and MiEn-

CP levels	MiEn-LEDRAGON[1]	Gas kinetics[1]				Cumulative gas, mL/0.5 g DM
		a	b	c	|a| + b	
CP10	0%	− 3.28	92.44	0.035	95.72	86.20
	3%	− 6.36	113.41	0.030	119.77	100.90
CP12	0%	− 3.60	92.68	0.037	96.28	86.53
	3%	− 6.34	114.07	0.029	120.41	101.10
CP14	0%	− 3.51	92.61	0.035	96.12	86.00
	3%	− 6.15	113.95	0.030	120.10	101.40
CP16	0%	− 3.60	93.04	0.036	96.64	86.70
	3%	− 6.12	114.16	0.030	120.28	101.60
SEM		0.106	0.348	0.0005	0.345	0.369
Comparison						
	CP levels	0.48	0.31	0.43	0.21	0.44
	CP10	− 4.82	102.93	0.033	107.75	93.55
	CP12	− 4.97	103.38	0.033	108.35	93.82
	CP14	− 4.83	103.28	0.033	108.11	93.70
	CP16	− 4.86	103.60	0.033	108.46	94.15
	MiEn-LEDRAGON	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
	0%	-3.50^{a}	92.69^{b}	0.036^{a}	96.19^{b}	86.36^{b}
	3%	-6.24^{b}	113.90^{a}	0.030^{b}	120.14^{a}	101.25^{a}
	Interaction	0.09	0.92	0.12	0.89	0.69

Table 2. Influence of crude protein levels combined with microencapsulated phytonutrients pellet product (MiEn-LEDRAGON) supplementation on gas kinetics and cumulative gas at 96 h after incubation. [a–b] Value on the same column with different superscripts differ ($p < 0.05$); SEM = standard error of the mean; [1]a = the gas production from the immediately soluble fraction (mL/0.5 g DM), b = the gas production from the insoluble fraction (mL/0.5 g DM), c = the gas production rate (mL/h), |a| + b = the gas potential extent of gas production; MiEn-LEDRAGON = microencapsulation of phytonutrients pellet product from lemongrass mixed dragon fruit peel; CP10 = concentrate diet containing 10% DM of crude protein; CP12 = concentrate diet containing 12% DM of crude protein; CP14 = concentrate diet containing 14% DM of crude protein; CP16 = concentrate diet containing 16% DM of crude protein.

LEDRAGON supplementation, nor was there an effect by the CP levels factor, on cumulative gas production or any parameters of gas kinetics ($p > 0.05$). Meanwhile, the main factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate) decreased the soluble fraction of gas production (a) and increased the insoluble fraction of gas production (b) ($p < 0.05$). Additionally, the main factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate) exhibited a decrease in the rate of gas production value (c) ($p < 0.05$), which ranged from 0.029 to 0.037 mL/h. Furthermore, the 3% DM of MiEn-LEDRAGON supplementation in the total substrate exhibited an increase in cumulative gas production ranging from 86.00 to 101.60 mL/0.5 g DM of substrate (Fig. 1), as well as a higher the potential extent of gas (|a|+b) value ($p < 0.05$).

In vitro degradability, ammonia-nitrogen concentration, and pH value

Table 3 shows the influence of CP levels combined with MiEn-LEDRAGON supplementation on in vitro degradability, pH value, and NH_3-N concentration. There was no interaction effect between CP levels and MiEn-LEDRAGON supplementation on any parameters, nor were there changes in the IVDMD and IVOMD at 12 h of incubation ($p > 0.05$). Whereas the higher IVDMD and IVOMD at 24 h were observed in the main factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate) ($p < 0.05$). The in vitro pH value at 12 h of incubation exhibited a higher value by the factor of crude protein levels (14 or 16% DM in the concentrate diet), and NH_3-N concentration at 12 h of incubation increased due to the factor of CP levels (16% DM in the concentrate diet) ($p < 0.05$). While the in vitro pH value and NH_3-N concentration at 24 h of incubation were increased by the main factor of CP levels (16% DM in the diet) and the MiEn-LEDRAGON supplementation (3% in the total DM substrate).

In vitro microbial dynamics

The influence of CP levels combined with MiEn-LEDRAGON supplementation on microbial dynamics are shown in Table 4. There was no interaction effect between CP levels and MiEn-LEDRAGON supplementation on the microbial population and the number of *M. elsdenii* ($p > 0.05$). The *F. succinogenes* population at 12 h of incubation showed a greater number ($p < 0.05$) due to the factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate). Similarly, the main factor of CP levels (12% DM in the concentrate diet or higher) and supplementation with 3% of MiEn-LEDRAGON in the total DM substrate influenced a higher number of *F. succinogenes* at 24 h of incubation ($p < 0.05$). Whereas there was a lower number of *R. flavefaciens*, *B. fibrisolvens*, and *Methanobacteriales* due to supplementation with 3% of MiEn-LEDRAGON in the total DM substrate

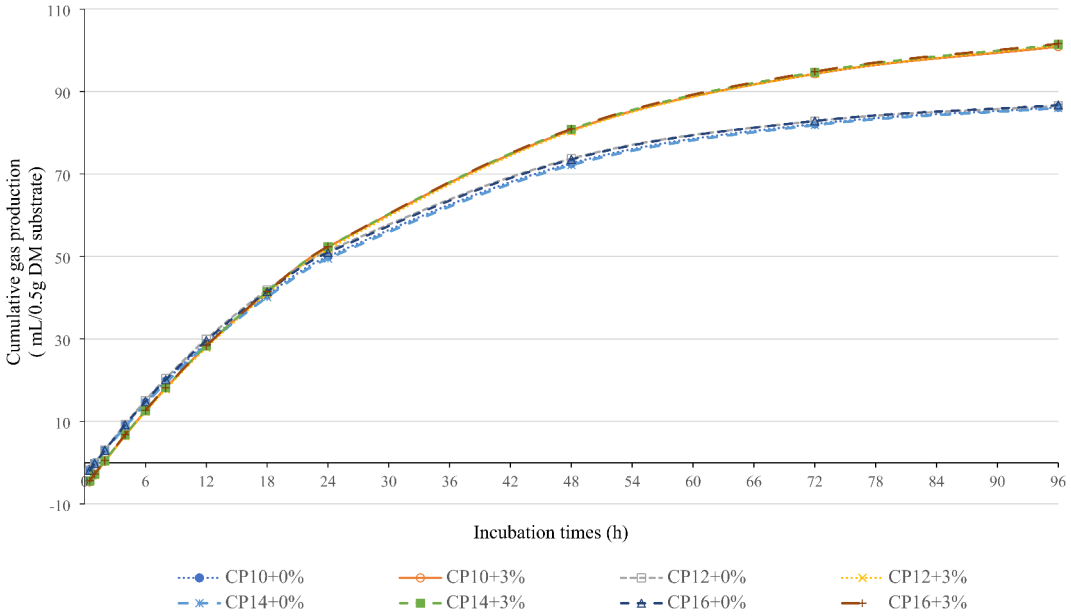

Fig. 1. Influence of crude protein levels combined with microencapsulated phytonutrients pellet product (MiEn-LEDRAGON) supplementation on in vitro cumulative gas production during incubation times.

CP levels	MiEn-LEDRAGON	IVDMD, %		IVOMD, %		Ammonia-nitrogen, mg/dL		pH value	
		12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
CP10	0%	49.21	54.90	52.81	59.61	9.62	11.89	6.80	6.72
	3%	49.50	56.31	53.49	60.29	9.65	12.13	6.81	6.71
CP12	0%	49.32	55.33	52.90	59.58	12.32	13.77	6.81	6.72
	3%	49.42	56.41	53.11	60.39	12.17	14.19	6.81	6.73
CP14	0%	49.20	55.12	53.58	59.70	14.03	15.82	6.86	6.74
	3%	49.51	56.18	52.92	60.49	13.62	17.02	6.85	6.75
CP16	0%	49.21	54.89	53.51	59.91	15.50	17.70	6.87	6.74
	3%	49.73	56.41	53.39	61.19	15.70	18.68	6.86	6.75
SEM		0.34	0.32	0.46	0.42	0.19	0.281	0.006	0.013
Comparison									
	CP levels	0.98	0.86	0.80	0.50	< 0.01	< 0.01	< 0.01	< 0.01
	CP10	49.36	55.61	53.15	59.95	9.64^{d}	12.01^{d}	6.81^{b}	6.72^{c}
	CP12	49.37	55.87	53.01	59.99	12.25^{c}	13.98^{c}	6.81^{b}	6.73^{bc}
	CP14	49.36	55.65	53.25	60.10	13.83^{b}	16.42^{b}	6.86^{a}	6.74^{b}
	CP16	49.47	55.65	53.45	60.55	15.60^{a}	18.19^{a}	6.87^{a}	6.75^{a}
	MiEn-LEDRAGON	0.21	< 0.01	0.99	< 0.01	0.49	< 0.01	0.47	< 0.01
	0%	49.24	55.06^{b}	53.20	59.70^{b}	12.87	14.80^{b}	6.84	6.73^{b}
	3%	49.54	56.33^{a}	53.23	60.59^{a}	12.79	15.51^{a}	6.83	6.74^{a}
	Interaction	0.95	0.95	0.52	0.86	0.48	0.30	0.78	0.07

Table 3. Influence of crude protein levels combined with microencapsulated phytonutrients pellet product (MiEn-LEDRAGON) supplementation on in vitro degradability, ammonia-nitrogen concentration, pH value. $^{a–d}$ Value on the same column with different superscripts differ ($p < 0.05$); SEM = standard error of the mean; MiEn-LEDRAGON = microencapsulation of phytonutrients pellet product from lemongrass mixed dragon fruit peel; CP10 = concentrate diet containing 10% DM of crude protein; CP12 = concentrate diet containing 12% DM of crude protein; CP14 = concentrate diet containing 14% DM of crude protein; CP16 = concentrate diet containing 16% DM of crude protein; IVDMD = in vitro dry matter degradability; IVOMD = in vitro organic matter degradability.

CP levels	MiEn-LEDRAGON	Log$_{10}$ copies/mL of rumen content													
		F. succinogenes		*R. albus*		*R. flavefaciens*		*M. elsdenii*		*B. fibrisolvens*		*B. proteoclasticus*		Methanobacteriales	
		12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
CP10	0%	9.32	9.67	9.40	10.16	9.46	10.33	11.00	11.13	10.18	10.69	8.62	8.79	9.18	9.98
	3%	9.54	10.00	9.33	10.06	9.34	10.28	11.07	11.15	10.28	10.75	8.93	8.88	8.87	9.52
CP12	0%	9.41	9.89	9.52	10.17	9.51	10.34	11.06	11.14	10.18	10.73	8.77	8.85	9.29	9.82
	3%	9.72	9.98	9.18	10.11	9.37	10.28	10.98	11.09	10.31	10.83	8.95	8.86	9.11	9.57
CP14	0%	9.56	10.02	9.50	10.16	9.49	10.35	11.04	11.12	10.26	10.71	8.75	8.85	9.29	9.79
	3%	9.75	10.18	9.25	10.10	9.35	10.29	11.30	11.11	10.30	10.74	8.96	8.90	9.04	9.65
CP16	0%	9.54	10.04	9.53	10.18	9.54	10.37	11.00	11.13	10.15	10.73	8.75	8.84	9.24	9.80
	3%	9.64	10.15	9.43	10.14	9.40	10.30	11.02	11.12	10.30	10.70	8.97	8.86	9.10	9.64
SEM		0.103	0.116	0.102	0.034	0.078	0.028	0.081	0.062	0.045	0.092	0.123	0.091	0.095	0.129
Comparison															
	CP levels	0.24	0.03	0.57	0.56	0.84	0.64	0.25	0.54	0.66	0.89	0.87	0.96	0.34	0.97
	CP10	9.43	9.84^{b}	9.37	10.11	9.40	10.31	11.03	11.14	10.23	10.72	8.78	8.84	9.03	9.75
	CP12	9.57	9.94^{ab}	9.35	10.14	9.44	10.31	11.02	11.12	10.25	10.78	8.86	8.86	9.20	9.70
	CP14	9.66	10.10^{a}	9.37	10.13	9.42	10.32	11.17	11.12	10.28	10.73	8.86	8.88	9.17	9.72
	CP16	9.59	10.10^{a}	9.48	10.16	9.47	10.34	11.01	11.13	10.23	10.72	8.86	8.85	9.17	9.72
	MiEn-LEDRAGON	0.02	0.02	0.03	0.03	0.04	0.02	0.25	0.50	0.01	0.56	0.03	0.52	0.01	0.02
	0%	9.46^{b}	9.91^{b}	9.49^{a}	10.17^{a}	9.50^{a}	10.35^{a}	11.03	11.13	10.19^{b}	10.72	8.72^{b}	8.83	9.25^{a}	9.85^{a}
	3%	9.66^{a}	10.08^{a}	9.30^{b}	10.10^{b}	9.37^{b}	10.29^{b}	11.09	11.12	10.30^{a}	10.76	8.95^{a}	8.88	9.03^{b}	9.60^{b}
	Interaction	0.78	0.44	0.53	0.78	0.99	0.98	0.30	0.83	0.67	0.91	0.96	0.97	0.81	0.59

Table 4. Influence of crude protein levels combined with microencapsulated phytonutrients pellet product (MiEn-LEDRAGON) supplementation on in vitro microbial population. [a–c] Value on the same column with different superscripts differ ($p < 0.05$); SEM = standard error of the mean; MiEn-LEDRAGON = microencapsulation of phytonutrients pellet product from lemongrass mixed dragon fruit peel; CP10 = concentrate diet containing 10% DM of crude protein; CP12 = concentrate diet containing 12% DM of crude protein; CP14 = concentrate diet containing 14% DM of crude protein; CP16 = concentrate diet containing 16% DM of crude protein.

($p < 0.05$). The populations of *R. flavefaciens* and *B. fibrisolvens* at 12 h of incubation were increased ($p < 0.05$) by the main factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate), whereas similar populations of both were observed at 24 h of incubation ($p > 0.05$).

In vitro volatile fatty acids concentration and methane production

Table 5 shows the influence of CP levels combined with MiEn-LEDRAGON supplementation on in vitro VFA concentration and CH_4 production. There was no interaction effect between CP levels and MiEn-LEDRAGON supplementation, nor was there an effect by the main factor of CP levels on total VFA concentration, proportions of VFA, and CH_4 production ($p > 0.05$). There was no notable variation in total VFA concentration at 12 h of incubation among the different dietary treatments ($p > 0.05$). However, total VFA concentration at 24 h of incubation was significantly higher ($p < 0.05$) due to the main factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate). The proportion of C2 decreased ($p < 0.05$) due to MiEn-LEDRAGON supplementation (3% in the total DM substrate). Meanwhile, the proportion of C3 increased as a result of MiEn-LEDRAGON supplementation ($p < 0.05$). Furthermore, MiEn-LEDRAGON supplementation resulted in a higher proportion of C4 at 12 h ($p < 0.05$), whereas the proportion of C4 at 24 h of incubation remained similar ($p > 0.05$). Additionally, MiEn-LEDRAGON supplementation resulted in a lower CH_4 production ($p < 0.05$).

Discussion

Gas production and gas kinetics

In this study, cumulative gas production and gas kinetics were unaffected by CP levels, which could be due to the fact that protein fermentation was not a main contributor to gas production[38–40]. This finding is consistent with previous studies[40,41], which demonstrated that the varying CP levels (10 to 16% DM in the diet) did not significantly affect gas production and the rate of gas. Meanwhile, the main factor of MiEn-LEDRAGON supplementation had an impact on changes in cumulative gas production and gas kinetics. In this study, the soluble fraction (a) of kinetic gas demonstrated a negative value according to the exponential mathematical models of gas kinetics output, which reflect the relationship between substrates and microbial activity[38–40]. This indicates the rapid initial growth and development phase of ruminal microbes on the diet surfaces, which occurs during the early phase of the incubation process[38]. It appears as though there is a gap period after the soluble portion of the diet is utilized before the cell walls start to ferment[5,39]. Therefore, several studies suggested an effective way to describe and optimally represent the (a) value by using the absolute value (|a|)[5,40,43–45]. Moreover,

CP levels	MiEn-LEDRAGON	TVFA, mmol/L		C2, %		C3, %		C4, %		CH_4, mL/0.5 g DM substrate	
		12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
CP10	0%	61.04	76.20	68.55	65.01	22.52	24.44	8.93	10.55	2.99	7.21
	3%	60.46	82.16	65.42	62.04	25.08	26.95	9.50	11.01	2.72	6.68
CP12	0%	59.44	76.13	67.11	64.63	23.75	24.35	9.14	11.02	3.06	7.38
	3%	61.62	81.44	66.23	61.94	24.42	26.97	9.35	11.09	2.78	6.70
CP14	0%	61.46	76.98	66.28	64.13	24.47	25.05	9.25	10.82	2.96	7.34
	3%	59.60	80.44	65.23	61.41	25.26	27.42	9.51	11.17	2.77	6.76
CP16	0%	60.37	75.86	66.73	64.41	24.20	24.59	9.07	11.00	2.94	7.27
	3%	60.63	81.88	65.40	63.17	25.36	26.07	9.24	10.76	2.76	6.72
SEM		1.575	0.745	0.531	0.710	0.434	0.473	0.147	0.374	0.051	0.085
Comparison											
	CP levels	0.99	0.92	0.20	0.55	0.17	0.34	0.51	0.88	0.49	0.58
	CP10	60.75	79.18	66.99	63.53	23.80	25.70	9.22	10.78	2.86	6.95
	CP12	60.53	78.79	66.67	63.29	24.09	25.66	9.25	11.06	2.92	7.04
	CP14	60.53	78.71	65.76	62.77	24.87	26.24	9.38	11.00	2.87	7.05
	CP16	60.50	78.87	66.07	63.79	24.78	25.33	9.16	10.88	2.85	7.00
	MiEn-LEDRAGON	0.98	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.02	0.56	< 0.01	< 0.01
	0%	60.58	76.29^{b}	67.17^{a}	64.55^{a}	23.74^{b}	24.61^{b}	9.10^{b}	10.85	2.99^{a}	7.30^{a}
	3%	60.58	81.48^{a}	65.57^{b}	62.14^{b}	25.03^{a}	26.85^{a}	9.40^{a}	11.01	2.76^{b}	6.72^{b}
	Interaction	0.64	0.35	0.11	0.6242	0.10	0.62	0.54	0.79	0.72	0.80

Table 5. Influence of crude protein levels combined with microencapsulated phytonutrients pellet product (MiEn-LEDRAGON) supplementation on in vitro volatile fatty acid concentrations and methane production. $^{a–b}$ Value on the same column with different superscripts differ ($p < 0.05$); SEM = standard error of the mean; MiEn-LEDRAGON = microencapsulation of phytonutrients pellet product from lemongrass mixed dragon fruit peel; CP10 = concentrate diet containing 10% DM of crude protein; CP12 = concentrate diet containing 12% DM of crude protein; CP14 = concentrate diet containing 14% DM of crude protein; CP16 = concentrate diet containing 16% DM of crude protein; TVFA = total volatile fatty acid; C2 = acetic acid proportion; C3 = propionic acid proportion; C4 = butyric acid proportion; CH_4 = methane production.

the higher |a| value shown after taking MiEn-LEDRAGON supplements may be due to the soluble dietary component of oligosaccharides, specifically fructooligosaccharides, which are obtained from the dragon fruit peel extract present in MiEn-LEDRAGON[5]. These oligosaccharides (soluble dietary fraction) enhance the ability to attach to ruminal microorganisms, thereby increasing gas production[5,38]. Suriyapha et al.[5] demonstrated that supplementation with MiEn-LEDRAGON increases the |a| value of in in vitro gas study.

In the current study, the main factor of MiEn-LEDRAGON supplementation showed the higher level of insoluble gas production (b). This probably be due to the higher level of insoluble fractions from protein-based polymers that make up the wall materials or carrier in MiEn-LEDRAGON[5]. In the microencapsulation process, proteins are one type of material that typically serves as wall carriers, allowing the core substrate to remain in its native state under challenging environmental conditions. In most cases, they are insoluble or bonded without significant degradation; however, when exposed to diluted acids or bases, they can dissolve in small amounts[46]. Thus, one possible explanation for the greater (b) value of gas is that the increased insoluble content from the protein wall material contributes to this effect[5]. In previous studies[47,48] revealed that supplementation with phytonutrients encapsulation product in the diet had an impact on higher (b) value of gas. Similarly, Suriyapha et al.[5] indicated that supplementation with MiEn-LEDRAGON increases (b) value of gas in in vitro study.

Usually, a rapid rate of gas production (c) correlates with a higher the soluble fraction[38,39,43,44]. However, the current study observed a lower (c) value by the main factor of MiEn-LEDRAGON supplementation. The possible cause of this effect is the fact that MiEn-LEDRAGON contains an increased number of protein wall constituents, which are insoluble components[5,47]. The degradation of the insoluble proportion usually occurs four times slower than that of the soluble proportion during anaerobic fermentation, resulting in lower microbial activity and a reduced degradation rate[49]. Consequently, this extends the stability and persistence of the core substrate under the ruminal anaerobic fermentation[5,50]. Recent studies[5,47,51] revealed that the supplementation of phytonutrient encapsulation products decreases the gas production rate in in vitro studies. Therefore, microencapsulated phytonutrients effectively protect bioactive compounds, resulting in a slower fermentation phase or degradation rate until the compounds reach their intended target.

In this study, the main factor of MiEn-LEDRAGON supplementation increased the potential gas production (|a| + b), which could be attributed to higher values for both (|a|) and (b), suggesting and estimating the fermentation of carbohydrates into VFAs[39,40]. Suriyapha et al.[5] demonstrated that supplementation with MiEn-LEDRAGON increases the (|a| + b) value of gas in in vitro study. In the present study, the main factor of MiEn-LEDRAGON supplementation showed a greater cumulative gas at 96 h after incubation which may be linked

to its total flavonoid and phenolic content, along with its antioxidative properties in MiEn-LEDRAGON. The phytonutrient microencapsulation products are rich in these components, which could improve the accessibility and utilization of the diet, thereby enhancing microbial activity, gas production, and in vitro fermentation[5,7,48,51].

In vitro degradability, ammonia-nitrogen concentration, and pH value

In this study, the results revealed that the main factor of CP levels had no influence on IVDMD and IVOMD. These findings are in line with previous studies[42,52], which reported that increasing CP levels in the diet did not affect in vitro degradability or apparent digestibility. However, these results differ from other studies[53–55], which noted that CP levels did have an impact on digestibility and that optimal CP levels in the concentrate diet could promote ruminal degradability and digestibility. These varying results suggest that the effect of CP levels on nutrient digestibility differs and may be influenced by factors such as the protein source or the balance of rumen-undegradable protein (RUP) and rumen-degradable protein (RDP) in the diet[41,56]. Meanwhile, a higher IVDMD and IVOMD at 24 h were observed by the main factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate), which could be due to MiEn-LEDRAGON being rich in flavonoids and phenolic compounds, which exert various biological effects on ruminal microbes and microbial activities, leading to improved degradability[5,48,57]. Moreover, this effect could be due to MiEn-LEDRAGON containing balanced levels of condensed tannins, saponins, and essential nutrients that promote microbial growth and activity, thereby positively influencing ruminal fermentation and enhancing nutrient degradability[5,12,58]. Suriyapha et al.[5] demonstrated that greater in vitro degradability was observed with the supplementation of MiEn-LEDRAGON at 3% in the total DM substrate. Similarly, the previous study of Matra et al.[48] demonstrated higher in vitro degradability when the diet was supplemented with the phytonutrients microencapsulation product (*Mitragyna* leaf extracts).

In the current study, NH_3-N concentration was increased by the main factor of CP levels, with higher CP levels in the concentrate diet. This probably be due to the higher CP level stimulating proteolytic microbial activity, as well as the adjustment of urea levels to balance the CP in the diet. This causes microbial enzymes to catalyze the hydrolysis of NPN-urea, leading to the production of ruminal NH_3-N and subsequently increasing its concentration[44,59,60]. Furthermore, the main factor of MiEn-LEDRAGON supplementation (3% in the total DM substrate) resulted in higher NH_3-N concentration at 24 h of incubation. This could be due to the high CP content of the cricket protein extract serving as the wall carrier material in MiEn-LEDRAGON[5]. Moreover, it could be due to the ability of phytonutrient active compounds extracted from plants to promote the proteolysis process[48]. Suriyapha et al.[5] revealed that the supplementation of MiEn-LEDRAGON influenced an increase NH_3-N concentration in an in vitro study. Similarly, Matra et al.[48] demonstrated a higher in vitro NH_3-N concentration when the diet was supplemented with a microencapsulated phytonutrient product (*Mitragyna* leaf extracts). Typically, a minimum concentration of 50 mg/L NH_3-N is essential for ruminal microbial protein synthesis[61]. Since the results from this study showed NH_3-N levels well above that threshold, it can be inferred that the rumen microbiota had an adequate supply of NH_3-N to support efficient microbial protein synthesis.

In this study, the increase in in vitro pH values was influenced by the main factors of CP levels and MiEn-LEDRAGON supplementation. This may be due to the naturally alkaline nature of NH_3-N, which could accumulate and make the ruminal environment more alkaline, leading to a shift in the rumen's balance and causing the elevated ruminal pH[60,62].

Microbial changes

In this study, despite the variation in CP levels in the diets, it is noteworthy that the main effect of CP levels was observed only on the *F. succinogenes* population at 24 h of incubation, with no significant influence on the numbers of other species. This could be attributed to competition with other microflora for the availability of sufficient NH_3-N as nitrogen sources for microbial access and protein synthesis[61]. The previous studies[54,55,63] reported that increasing CP levels in the diet can enhance the ruminal microbe population and cellulolytic bacterial count. However, other studies[42,56,64–66] reported that varying CP levels did not significantly affect the cellulolytic microbial community or the population of dominant microflora. These inconsistencies in the effects of varying CP levels on microbial populations may be explained by variations in the animals and diet composition, particularly the levels of RDP and RUP in the diet as well as availability of sufficient nitrogen sources[55,56,61]. Meanwhile, the results of this study revealed that the main factor of MiEn-LEDRAGON supplementation potentially affects microbial diversity, particularly the populations of major cellulolytic bacteria (*F. succinogenes*, *R. albus*, and *R. flavefaciens*) and methanogens (*Methanobacteriales*). These compounds work by disrupting cytoplasmic membrane function, interfering with microbial cell wall formation, or obstructing nucleic acid synthesis in bacterial cells[67,68]. The differing sensitivities of bacterial outer membranes—Gram-positive bacteria (*Ruminococcus* species) being more affected than Gram-negative bacteria (*F. succinogenes*)—likely contributed to the decrease in *R. albus* and *R. flavefaciens* populations, with a compensatory rise in *F. succinogenes*[1,5,10]. Previous studies[1,69] have demonstrated that plant secondary compounds, such as tannins or flavonoids, can increase *F. succinogenes* populations while decreasing *R. albus* and *R. flavefaciens* populations. Similarly, Suriyapha et al.[5] reported that supplementing MiEn-LEDRAGON at 3% in the total DM substrate led to a rise in *F. succinogenes* and a reduction in *R. albus* and *R. flavefaciens* populations in in vitro study. Furthermore, this study revealed that the population of *B. fibrisolvens* and *B. proteoclasticus* at 12 h was increased by the main factor of MiEn-LEDRAGON supplementation. It is possible that MiEn-LEDRAGON, which is rich in plant-derived bioactive compounds, is stimulating and responsible for growing and enhancing the number of bacteria involved in the breakdown of cellulose and protein[5,70]. Zhan et al.[70] demonstrated that the use of alfalfa flavonoids in the diet of dairy cows could promote the growth of the ruminal *B. fibrisolvens* population in an in vivo study. Matra et al.[48] reported similar results, where supplementation with 6% of a microencapsulated phytonutrient product (*Mitragyna* leaf extracts) in the total DM substrate led to a higher

population of *B. fibrisolvens* in an in vitro study. Similarly, a recent study by Suriyapha et al.[5] indicated that supplementation with 3% of MiEn-LEDRAGON in the total DM substrate could promote the population of *Butyrivibrio* species. Moreover, this study also found a reduction in the *Methanobacteriales* (methanogen group) with the main factor of MiEn-LEDRAGON supplementation. This effect could be attributed to the phytonutrient or plant secondary active contents present in MiEn-LEDRAGON[5]. These plant derived secondary compounds can affect rumen methanogens by interacting with protein-based adhesins, inhibiting methanogen growth, disrupting protozoa- methanogen interactions, and reducing hydrogen (H) transfer, ultimately leading to lower methanogenesis and a decrease in ruminal CH_4 production[1,71,72]. Moreover, recent studies[47,48,51] have reported that microencapsulated plant bioactive compounds can reduce the *Methanobacteriales* population. Similarly, Suriyapha et al.[5] demonstrated that supplementing with 3% of MiEn-LEDRAGON in the total DM substrate could reduce the *Methanobacteriales* population without negatively affecting in vitro fermentation.

In vitro volatile fatty acid concentrations and methane production

In this study, the total VFA and individual VFA proportions (C2, C3, and C4) as well as CH_4 production were not impacted by the CP levels. This may be attributed to the similarity in carbohydrate sources (both structural and non-structural carbohydrates) across the experimental diets. Carbohydrate source is crucial for fermentation process and VFA production, particularly for C3 and the relationship between VFA and CH_4 production[40–42]. Additionally, this could be due to the similar influence on the microbial population by the main factor of CP levels, which microbial dynamic is important for VFA and CH_4 genesis[41,66]. Previous studies[41,42,63,73] have reported that variations in CP levels had no significant impact on VFA concentration or CH_4 production, which aligns with the observations in this study. While this finding demonstrated that the main factor of MiEn-LEDRAGON supplementation potentially affects VFA and CH_4 production. The main factor of MiEn-LEDRAGON supplementation shown in higher total VFA levels at 24 h of incubation, which is likely due to improved degradability[5]. Additionally, the secondary plant compounds present in MiEn-LEDRAGON probably supported microbial fermentation, thereby boosting the concentration of VFA[6,11,13]. Matra and Wanapat[12] found that pellets containing phytonutrients from dragon fruit peel improved the overall production of VFA. Similarly, Suriyapha et al.[5] suggested that 3% of MiEn-LEDRAGON supplementation in the total DM substrate enhances the total in vitro VFA concentration. Moreover, the study results demonstrated that the main factor of supplementation with MiEn-LEDRAGON resulted in an increased C3 proportion, alongside a reduced C2 proportion, and decreased CH_4 production. Furthermore, under conditions of excess hydrogen, plant secondary compounds may influence C3 production by using hydrogen (H) to produce C3, rather than using it as the primary source for CH_4 production[2,10]. The augmented C3 synthesis resulting from MiEn-LEDRAGON may be linked to an elevated abundance of *F. succinogenes*, a microorganism that generates succinate, a precursor for C3[1]. The increase in C3 generation is facilitated by the succinate-to-propionate conversion pathway, which is a significant method for generating C3 in the rumen[74]. Additionally, a lower number of *R. albus* and *R. flavefaciens*, which are significant producers of H and contribute to CH_4 production when interacting with methanogens[1,75], may also play a role in the observed effects. Conversely, the increase in *F. succinogenes*, which does not produce H, promotes higher C3 production and lower CH_4 generation and output[1,76]. Suriyapha et al.[5] also suggested that the supplementation of MiEn-LEDRAGON at 3% in the total DM substrate could enhance C3 production and decrease C2 and CH_4 production. Similarly, Matra and Wanapat[12] showed that the supplementation with pellet products form dragon fruit peel, rich in phytonutrients, led to a higher C3 proportion and a reduction in both C2 and CH_4 production. Moreover, this study observed an increase in C4 proportion at 12 h of incubation, which may be attributed to the rise in the populations of *B. fibrisolvens* and *B. proteoclasticus*, both key bacteria that produce C4 in rumen[77]. Suriyapha et al.[5] demonstrated that supplementing the diet with 3% of MiEn-LEDRAGON in the total DM substrate increases in vitro C4 production. Similarly, Matra et al.[48] showed a greater proportion of C4 when microencapsulated secondary plant active compounds were added to the diet.

Conclusion

In conclusion, this study found no interaction effect between CP levels and MiEn-LEDRAGON supplementation on any of the observed parameters. Increasing CP levels in the concentrate diet did not negatively impact gas production, fermentation characteristics, end-product formation, or microbial changes, while enhancing in vitro pH and NH_3-N levels. Furthermore, the supplementation of MiEn-LEDRAGON shows potential as a feed additive to improve ruminal fermentation characteristics, support microbial dynamics, and reduce methanogen populations and CH_4 production in ruminants. However, further evaluation and investigation of ruminal metabolism and in vivo trials are necessary to provide more in-depth information and confirm its benefits for animal production.

Data availability

The authors affirm that the data supporting this study's findings are included within the article. Additionally, the datasets generated and analyzed during the study can be obtained from the corresponding author upon reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

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