BMC Veterinary Research

RESEARCH

Open Access

Encapsulated phytogenic oils enhance in vitro rumen fermentation and reduce methane emissions

Srisan Phupaboon[1], Uswatun Muslykhah[1], Chaichana Suriyapha[1], Sukruthai Sommai[1], Maharach Matra[2], Gamonmas Dagaew[1], Rittikeard Prachumchai[3] and Metha Wanapat[1*]

Abstract

Background This research aimed to investigate bioaccessibility of garlic oil extract (GOE) and riceberry rice bran oil extract (RBRBOE) and to enhance the stability and delivery of plant-derived essential oils. Two EOs were used to formulate through encapsulation techniques of microencapsulated black soldier fly (BSF) protein with a GOE matrix, (mBSF-GOE), and a nanoemulsified RBRBOE, (nRBRBOE). A completely randomized design was used for the treatments, with various ratios of feed additives between the mBSF-GOE and nRBRBOE supplementations at 0:0, 6:0, 4:2, 2:4, and 0:6 mg in diet, with the R: C ratio at 60:40 using in vitro gas study.

Results The combination of mBSF-GOE and nRBRBOE at 4:2 mg for 12 and 24 h after fermentation had a significant impact on several factors ($p < 0.01$, < 0.05), including gas kinetics, cumulative gas production (96 h), in vitro dry matter degradability (IVDMD), and ruminal fermentation products. Specifically, the levels of propionate (C3) and total VFAs went up, while ruminal methane (CH_4) production decreased by 48.2%. Subsequently, there was no negative effect ($p > 0.05$) on the ruminal pH, ammonia nitrogen (NH_3-N) concentration, or the dynamics of the rumen microbiota population, while significantly decreasing the methanogen population in terms of *Methanobacteriales* (up to 3.3% after 24 h) ($p < 0.01$).

Conclusions Based on this study, it could be concluded that the supplementation of mBSF-GOE combined with nRBRBOE-based bioactive components could potentially be used as a ruminant feed enhancer to enhance fermentation efficiency and as technological feed additive substances to inhibit the methanogen population while mitigating CH_4 production.

Keywords Micro-nanocapsules, Feed delivery, Nutrients protection, Rumen fermentation, Methane emission, Garlic-in-riceberry

*Correspondence:
Metha Wanapat
metha@kku.ac.th
[1]Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Division of Animal Science, Department of Agricultural Technology, Faculty of Technology, Mahasarakham University, Maha Sarakham 44150, Thailand
[3]Department of Agricultural Technology, Faculty of Science and Technology, Thammasat University, Pathum Thani 12120, Thailand

Introduction

Due to 2050 the global population is projected to reach 9.6 billion, resulting in a doubling of purchasing power for meat and dairy products. Consequently, there will be a corresponding increase in greenhouse gas (GHG) emissions from livestock activities, particularly carbon dioxide (CO_2), nitrous oxide, and CH_4 [1]. Various natural herb essential oils (EOs) or tropical plant extracts, particularly garlic, lemongrass, and mangosteen peels, when fed, supply phytochemicals in terms of total polyphenolic substances such as condensed tannins (CTs), saponins (SPs), curcumin, quercetin, and other anthocyanins, which have been explored to decrease the production of CH_4 in the ruminant digestive system [2].

Previous research findings into the use of phytonutrients and bioactive components as feed additives, which is particularly relevant due to regulations regarding antibiotics and antimicrobial substances as well as mitigating CH_4 production in rumen fermentation. For instance, allicin (50.8 μg/g) from garlic (*Allium sativum*) essential oils [3] and anthocyanin and peonidin (2,316.7 and 245.7 μg/g) from riceberry rice (*Oryza sativa*) bran essential oils [4, 5]. The widely accepted notion is that the presence of feed additives containing phytogenic EOs has a substantial influence on the development of CH_4 production [6, 7]. This is primarily due to the actions on ruminal microbiota, which includes various species of protozoa and methanogenic archaea. These additives possess several antimicrobial properties, such as breaking down cell walls, enhancing membrane permeability, causing cytoplasmic coagulation, and damaging cytoplasmic membranes and membrane proteins [8]. Furthermore, numerous investigations have shown that EOs can be used as a substitute for antibiotics in animal feeds. However, their volatile nature, variable chemical composition, and bioactive compounds content, it is essential to achieve the required high-level stability and long-term release and act as a release controller during the ruminant's fermentation process [9, 10]. Encapsulation technology was designed to protect bioactive compounds, such as EOs from oxidative deterioration, resist high temperatures, enhance a rumen by-pass, and serve as an addition to ruminant feeding, aiming to increase effectiveness [11].

Microencapsulation and nanoemulsion are modern technologies using spray drying that are frequently employed in both human and animal nutrition to create stable products such as vitamins, minerals, polyunsaturated fatty acids, and other phytonutrients [12]. Both microencapsulation and nanoemulsion, are common techniques which employ different wall materials, such as chemical emulsifiers, isolated protein-based oils, and lipid matrix obtained from plants, animals, and insects, which have proven to be highly effective in delivering the product to the small intestine [12, 13]. According to Amin et al. [11], recent evidence suggests that the microencapsulated cinnamaldehyde EOs, namely Oilstat-G, significantly decreased in vitro fermentation parameters ruminal pH and total protozoa population while increasing the total VFAs content, leading to the conclusion that it was effective to ensure rumen by-pass, capable of enhanced rumen fermentation, and potentially mitigate CH_4 emission. Additionally, several studies investigating oil-in-water nanoencapsulation regarding three nanoemulsified EOs, namely olive oil, corn oil, and linseed oil, showed an ability to decrease the in vitro ruminal biohydrogenation of unsaturated fatty acid to saturated fatty acid without affecting the abundance of species of rumen microbiota [14]. Consequently, the hypothesis suggests that the micro/nanoparticles present in feed additives have a significant influence on the effectiveness of nutrient preservation, altering rumen fermentation, and decreasing CH_4 emissions.

Considering this, the purpose and aims of this in vitro gas study, were to evaluate the effects derived from supplementing with different ratios of mBSFGOE and nRBRBOE on improving ruminal fermentation in Thai cattle's rumen fluid. Furthermore, fermentation end-product characterization, particularly VFA production, CH_4 production, and the dynamics of the rumen microbiota population using real-time PCR techniques, were also evaluated.

Results

Nutritive vales and morphological characteristics of micro/nano-capsules

Table 1 shows the details of the feed ingredients, nutritive values, chemical composition, phytochemicals, and antioxidant components in both additives mBSF-GOE and nRBRBOE, respectively. In addition, the data in Table 1 presented the percentage of encapsulation efficiency (%EE) calculated from the TPC values obtained from internal encapsulated particles, which was 84.7% obtained from mBSF-GOE capsules and 67.7% in nRBRBOE particles, respectively.

As shown in Fig. 1, the morphology of the mBSF-GOE capsule is mostly round or irregularly shaped (Fig. 1a). It has a smooth surface alternating with the surrounding porous surface of the particle, which means that the particle sizes range from 12.6 to 19.2 μm. In addition, the nRBRBOE capsule (Fig. 1b) was entirely observed in spherical form and ranged from 1.1 to 5.0 μm, indicating a nanoparticle size.

In vitro gas production kinetics and nutrient degradability characteristics

The results of cumulative gas productions and in vitro dry matter degradability (IVDMD) of the present study

Table 1 Characterization of feed ingredients, chemical composition, and phytochemical components of additive

Items	Concentrate	Rice straw	mBFS-GOE	nRBR-BOE
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 mix[1]	1.0			
Chemical composition				
Dry matter (DM, %)	90.5	89.4	92.6	-
	----------% dry matter----------			
Organic matter (OM)	92.2	85.4	93.9	-
Crude protein (CP)	14.6	2.4	22.1	-
Neutral-detergent fiber (NDF)	20.5	78.9	17.4	-
Acid-detergent fiber (ADF)	8.2	52.6	23.2	-
Ether extract (%)			2.3	27.7
Phytonutrition content				
TPC (mg GAE/g DM or RO)	-	-	955.5	102.3
TFC (mg QUE/g DM or RO)	-	-	94.8	30.2
Total anthocyanin (μg Cy 3-glc/g RO)	-	-	-	90.1
Antioxidative values				
DPPH inhibition (%)	-	-	51.5	17.6
ABTS inhibition (%)	-	-	15.4	20.8
FRAP capacity (g TROE/kg DM)	-	-	17.3	11.0
Encapsulation efficiency (%)	-	-	84.7	67.7

mBSF-GOE, microencapsulated of black soldier fly-based protein extract mixed with garlic oil extract; nRBRBOE, nanoemulsified riceberry rice bran oil; RO, rice oil 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, garlic acid equivalent; QUE, quercetin equivalent; TROE, Trolox equivalent; Cy 3-glc, cyanidin 3-gluciside. [1] Mineral premix (contains per kg): vitamin A 10,000,000 IU; vitamin D 1,600,000 IU; vitamin E 70,000 IU; Fe 50 g; Mn 40 g; Zn 40 g; Cu 10 g; I 0.5 g; Se 0.1 g; Co 0.1 g

are displayed in Fig. 2; Table 2, these were significantly different between treatments ($p < 0.01$). Gas kinetics, encompassing gas production from the insoluble fraction (b), gas production rate constant for the insoluble fraction (c), potential extent of gas production (a + b), and cumulative gas at 96 h of post-fermentation, exhibited significant differences among treatments (quadratic and cubic; $p < 0.01$) due to the combined effects of mBSF-GOE and nRBRBOE supplementations. In contrast, the immediately soluble fraction (a) was significantly influenced (linear; $p < 0.01$). Interestingly, the combination supplementations increased ($p < 0.01$) the gas production rate constant for the insoluble fraction (c), with the highest values obtained from treatment (T3), while significantly decreasing ($p < 0.01$) the gas production from the insoluble fraction (b), the potential extent of gas production (a + b), and cumulative gas production was lower than other treatments (Fig. 2.)

Additionally, the effect of mBSF-GOE mixed with nRBRBOE supplementations in different feed treatments (T2, T3, T4, and T5) linearly increased ($p < 0.01$) the IVDMD values after 24 h post-fermentation. The treatment (T3) at 4:2 mg of supplementation had the highest values of IVDMD at 39.9% DM, as shown in Table 2.

Ruminal pH value and NH_3-N concentration

It can be seen from the data in Table 3 that the ruminal pH values after incubation at 12 h were affected ($p < 0.05$) by the combined supplementation in all treatments, while compared with the ruminal pH values at 24 h, they were not affected ($p < 0.05$). The mean ruminal pH values were not different for each treatment and fermentation time.

Moreover, the NH_3-N concentration (12 and 24 h) was not affected ($p < 0.05$) by neither levels of both mBSF-GOE and nRBRBOE supplementations (Table 3).

VFA profiles and CH_4 production

Treatment T3, with a supplementation ratio of 4:2 mg diet between mBSF-GOE and nRBRBOE, linearly increased ($p < 0.05$) the propionate (C_3) content and total VFA production. The same also recorded the highest total VFA production as T5 in comparison to the other treatments (T2 and T4) and the control group (T1) (Table 4). Additionally, the acetate (C_2) and acetate to propionate (C_2:C_3) contents were linearly decreased ($p < 0.05$). With respect to butyrate (C_4) content was not affected ($p > 0.05$) when compared within the treatment group of T3 and the control.

Moreover, Table 4, also shows the effect of a combination of mBSF-GOE and nRBRBOE supplementations on CH_4 production. The current results of the average amount of CH_4 production (at 24 h of fermentation) were steadily decreased (linear and quadratic; $p < 0.05$) due to increasing the level of feed additives in each treatment and fermentation time. Interestingly, the combination of both additives (mBSF-GOE and nRBRBOE) supplementation on the in vitro gas fermentation resulted in a 48.3% reduction in CH_4 production after 24 h post-fermentation, obtained from T3 at a ratio of 4:2 mg of diet. Furthermore, the results of this specific treatment at 12 and

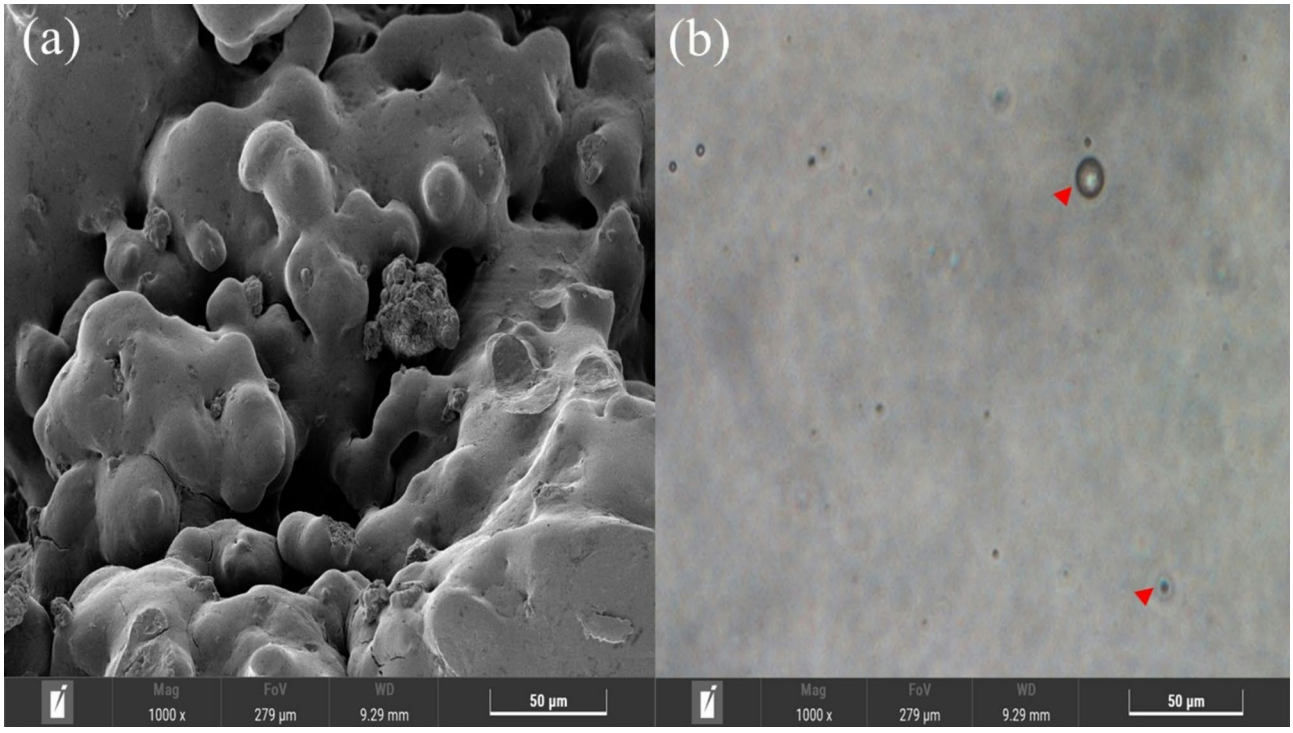

Fig. 1 FE-SEM micrographs showing the external micro and nano structures and surface morphological characteristics of mBSF-GOE (**a**) and nRBRBOE (**b**) particles as viewed at a scale bar at 50 µm

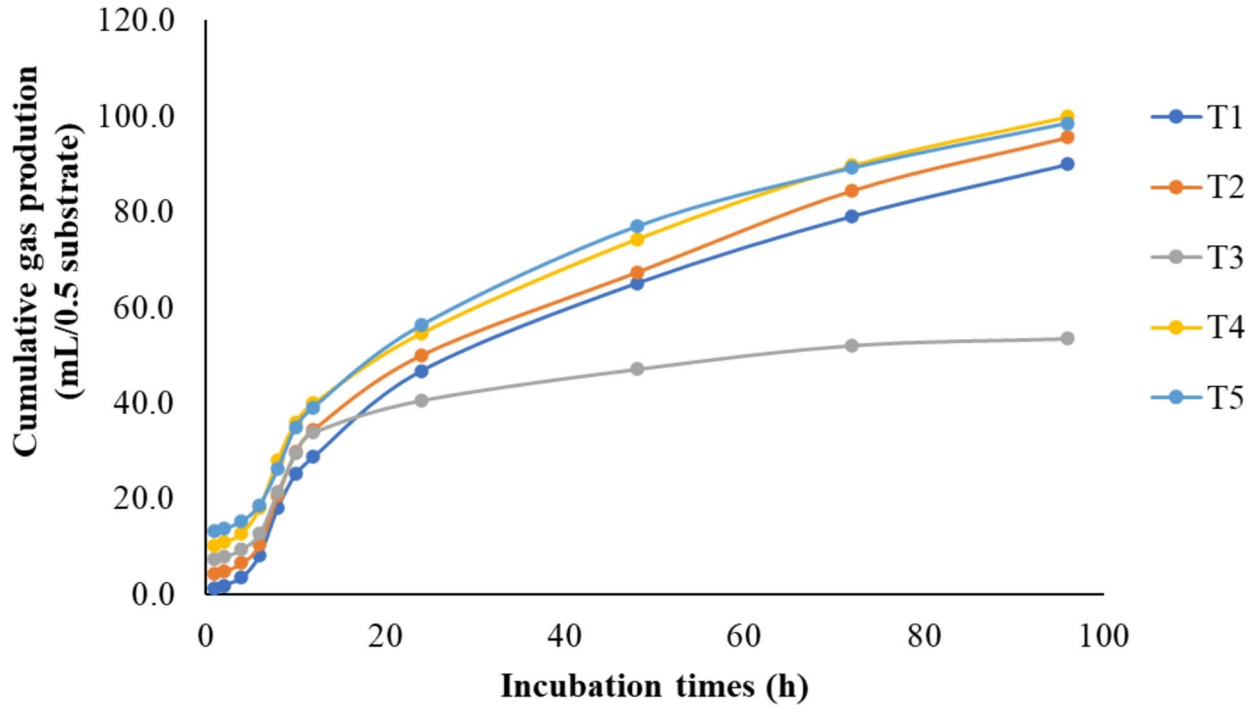

Fig. 2 The effect of mBSF-GOE and nRBRBOE supplementations at various treatment of T1: 0:0; T2: 6:0; T3: 4:2; T4: 2:4; T5: 0:6 based on the cumulative gas production curves throughout at 1–96 h incubation period

Table 2 Effect of microencapsulated black soldier fly-based protein extract (BSF) mixed with garlic oil extract (GOE) and nanoemulsified riceberry rice bran oil (nRBRBOE) ratio supplementation on kinetic of in vitro gas production and nutrient degradability

T[1]	mBSF-GOE to nRBRBOE ratios, (mg)	Gas kinetics[2]				Gas (96 h) mL/0.2 g DM	IVDMD (% DM)	
		a	b	c	a + b		12 h	24 h
T1	0:0	-3.3[b]	96.1[a]	0.029[b]	92.8[a]	89.9[a]	29.0	34.2[b]
T2	6:0	0.1[b]	98.5[a]	0.029[b]	98.6[a]	95.6[a]	27.2	37.6[a]
T3	4:2	0.9[b]	51.0[b]	0.067[a]	51.9[b]	53.4[b]	26.7	39.9[a]
T4	2:4	6.4[a]	96.2[a]	0.029[b]	102.6[a]	99.8[a]	25.9	39.6[a]
T5	0:6	7.9[a]	95.1[a]	0.029[b]	103.0[a]	98.5[a]	29.6	38.8[a]
SEM		0.41	1.95	0.08	1.83	1.59	0.79	0.80
Orthogonal polynomials								
Linear		< 0.01	0.04	0.01	0.37	0.45	0.347	< 0.01
Quadratic		0.28	< 0.01	< 0.01	< 0.01	< 0.01	0.826	0.03
Cubic		0.11	< 0.01	< 0.01	< 0.01	< 0.01	0.883	0.38

[1]T, each treatment in this experiment; [2] 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); IVDMD, in vitro dry matter degradability; SEM, standard error of mean; [a–b] 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 microencapsulated black soldier fly-based protein extract (BSF) mixed with garlic oil extract (GOE) and nanoemulsified riceberry rice bran oil (nRBRBOE) ratio supplementation on in vitro ruminal pH and ammonia nitrogen concentration

Treatment	mBSF-GOE to nRBRBOE ratios, (mg)	Ruminal pH		Ammonia nitrogen (mg/dL)	
		12 h	24 h	12 h	24 h
T1	0:0	6.99[a]	6.85	17.2	18.8
T2	6:0	6.93[b]	6.94	17.3	18.6
T3	4:2	6.93[b]	6.84	18.1	18.3
T4	2:4	6.94[ab]	6.83	18.0	18.1
T5	0:6	6.95[ab]	6.85	18.0	18.2
SEM		0.04	0.01	0.12	0.09
Orthogonal polynomials					
Linear		0.07	0.10	0.06	0.20
Quadratic		0.04	0.72	1.00	0.91
Cubic		0.49	0.88	0.34	0.97

[a–b] 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

24 h showed a lower value of CH_4 production than other treatments ($p < 0.05$) (Table 4).

Rumen microbiota population

As shown in Table 5, the effect of the combination of mBSF-GOE and nRBRBOE supplementations did not change the dynamics of the rumen microbiota population, namely cellulolytic bacteria (e.g., *F. succinogenes*, *R. albus*, and *R. flavefaciens)*, acidobacteria (e.g., *M. elsdenii* and *B. fibrisolvens*), as well as microbial synthesis protein and hydrogenation bacteria (e.g., *B. fibrisolvens and B. proteoclasticus*), which were not significantly different among treatments ($p > 0.05$) and fermentation times (12 and 24 h). Interestingly, the relative abundance of *Methanobacteriales* of specific species (e.g., *M. methylutens*, *M. stadtmanae*, and *M. furmicicum*) was significantly decreased (cubic effect; $p < 0.01$) with the amount of gene copy numbers due to the dose of feed additive of the combination of mBSF-GOE and nRBRBOE after incubation at 24 h. From this result, when comparing the amount of gene copy number of *Methanobacteriales* obtained from T3 and control experiments at 12 and 24 h after incubation times, it was found that their population could be reduced to 1.1% and 3.3%, respectively, as similar with T4 in ratio at a 2:4 mg of the mBSF-GOE combined with nRBRBOE supplementation ($p < 0.01$), as shown in Table 5.

Discussion

With regards to the in vitro kinetic gas production and their degradability characteristics, Molho-Ortiz et al. [15], explained that the inhibitory effect on rumen fermentation mediated by plant-essential oils (EOs) can be attributed to their chemical makeup. EOs are complex mixtures of secondary plant metabolites that exhibit a highly varied composition. There is speculation that their process includes disrupting the membrane of microbes [16]. These findings are consistent with our present study, which found that the effect of phytogenic oils: mBSF-GOE and nBRBROE, used as feed additives that contain phytonutrient-based bioactive components, such as TPC (e.g., condensed tannins; CTs) and TFC (e.g., saponins; STs), is throughout microencapsulation and nanoemulsion technologies. The most interesting finding was that these small particles of mBSF-GOE combined with nBRBROE (T3) at 4:2 mg of substrate supplemented on in vitro gas fermentation significantly affected on gas fermentation kinetics from the immediately soluble fraction (a, b, c, a + b, cumulative gas production at 96 h) and in vitro degradability in terms of IVDMD (%DM). The current results are similar to those of Kongmun et al. [17],

Table 4 Effect of microencapsulated black soldier fly-based protein extract (BSF) mixed with garlic oil extract (GOE) and nanoemulsified riceberry rice bran oil (nRBRBOE) ratio supplementation on in vitro volatile fatty acid (VFA) profiles, total VFA production, and methane (CH_4) production

Treatment	mBSF-GOE to nRBRBOE ratios, (mg)	VFA (mol/100 ml)			C_2:C_3 Ratio	Total VFA (mmol/L)	Methane production (%)	
		C_2	C_3	C_4			12 h	24 h
T1	0:0	69.5^{a}	20.4^{c}	10.2	3.4^{a}	104.1^{c}	16.6	19.4^{a}
T2	6:0	66.5^{c}	22.8^{b}	11.1	2.9^{ab}	121.8^{b}	16.3	7.0^{b}
T3	4:2	67.2^{b}	27.1^{a}	10.8	2.5^{c}	124.6^{b}	14.0	8.5^{b}
T4	2:4	66.5^{c}	22.5^{b}	11.0	3.0^{b}	124.2^{b}	12.9	8.3^{b}
T5	0:6	67.4^{b}	22.3^{b}	10.3	3.0^{b}	131.1^{a}	12.9	7.7^{b}
SEM		0.69	0.64	0.08	0.08	4.12	0.71	0.66
Orthogonal polynomials								
Linear		0.01	0.01	0.09	0.01	0. 03	0.44	< 0.01
Quadratic		0.32	0.21	0.13	0.34	0.46	0.50	0.02
Cubic		0.94	0.76	0.12	0.96	0.83	0.57	0.44

VFA, volatile fatty acids; C_2, acetate; C_3, propionate; C_4, butyrate; C_2:C_3, acetate to propionate ratio; $^{a–c}$ 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 5 Effect of microencapsulated black soldier fly-based protein extract (BSF) mixed with garlic oil extract (GOE) and nanoemulsified riceberry rice bran oil (nRBRBOE) ratio supplementation on rumen microbial population

Species	Times	mBSF-GOE to nRBRBOE ratios, (mg)					SEM	Orthogonal polynomials[1]		
		0:0	6:0	4:2	2:4	0:6		L	Q	C
F. succinogenes (Log copies/mL)	12 h	7.2	7.8	7.5	8.2	8.7	0.26	0.40	0.95	0.60
	24 h	9.6	9.9	9.2	7.7	8.6	0.21	0.05	0.20	0.96
R. albus (Log copies/mL)	12 h	8.8	8.5	8.9	8.6	9.0	0.06	0.85	0.89	0.10
	24 h	9.4	9.5	9.5	9.5	9.4	0.05	0.70	0.53	0.89
R. flavefaciens (Log copies/mL)	12 h	8.1	8.2	8.7	8.1	7.7	0.25	0.88	0.63	0.65
	24 h	8.5	7.8	9.2	10.0	9.6	0.22	0.08	0.25	0.37
M. elsdenii (Log copies/mL)	12 h	9.0	8.7	9.2	8.8	9.3	0.26	0.70	0.76	0.07
	24 h	9.1	9.4	8.8	8.7	8.9	0.09	0.23	0.50	0.31
B.fibrisolvens (Log copies/mL)	12 h	8.7	8.3	8.6	8.0	8.8	0.07	0.08	0.70	0.14
	24 h	8.7	8.9	8.3	8.8	8.8	0.07	0.74	0.50	0.08
B. proteoclasticus (Log copies/mL)	12 h	9.4	9.0	9.5	9.2	9.2	0.10	0.94	1.00	0.23
	24 h	9.3	9.1	9.4	9.4	9.0	0.04	0.47	0.42	0.23
Methanobacteriales (Log copies/mL)	12 h	9.3	9.2	9.2	9.2	9.2	0.03	0.43	0.55	0.78
	24 h	9.2^{a}	9.0^{b}	8.9^{b}	8.9^{b}	9.0^{b}	0.01	1.00	0.25	< 0.01

[1] L, linear; Q, quadratic; C, cubic; $^{a–b}$ 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

who discovered that adding different amounts of coconut oil and garlic powder had a big effect on different components of gas production, such as the amount of insoluble matter (b), the possible amount of gas production (a + b), the rate constants for gas production in the insoluble matter (c), the total amount of gas produced at 72 h, and the true digestibility in a laboratory setting. However, the intercept value (a) did not show significant differences between the different treatments [17]. This also accords with our earlier research from Phupaboon et al. [7], which found that the feed additive with a combination of chitosan-microencapsulated lemongrass oil extract combined with mangosteen peel extract (mLEMANGOS) retained the phytonutrient-based antimicrobial in terms of CT and SP contents at 2–6% of the total DM substrate. T3 resulted in an enhancement of the gas production rate constant for the insoluble fraction (c), decreasing cumulative gas at 96 h, and increasing the %DM of IVDMD values after 12 and 24 h of post-fermentation. Additionally, the present study seems to be consistent with previous research, which found that the interaction subsequently influences microbial activity by using phytonutrients or phytogenic oils, which possess the ability to interact with fiber and protein compounds [18]. Another piece of evidence from Phesatcha et al. [19] stated that nutritive values such as starch degradation under in vitro rumen fermentation may be influenced by its important role in regulating energy usage for the growth of rumen microbes, increasing the number of rumen microbiome

populations, in particular cellulolytic bacteria, and aiding in the digestion of feed.

Under this study, in vitro fermentation characteristics, end-products, and ruminal microbiota consortia were investigated. Numerous researches have attempted to explain in vitro studies on the supplementation of rumen-protected using microencapsulated EOs from linseed oil on rumen fermentation characteristics, fermentation end-products, and mitigation of CH_4 emission [12]. In addition, in a recent study by Amin et al. [11], the in vitro ruminal degradability of microencapsulated EOs containing cinnamldehyde plus vitamins, namely, Olistat-G was discovered to be highly effective in protecting them from degradation in the rumen, therefore ensuring rumen by-pass. In the fermentation profile at 0 h, it was seen that Olistat-G caused a significant decrease in ruminal pH and the total number of protozoa after 48 h. However, there was an increase in the total VFAs.

In this study, the combined supplementation of mBSF-GOE and nBRBROE supplementations, as feed additive, improved the ruminal pH with different treatments. This result was in agreement with Wanapat et al. [20] and Matra et al. [9], who reported that the optimal ruminal pH for typical rumen fermentation, rumen microbiome growth, particularly cellulolytic bacteria, and their activity to degrade nutritional composition in feed ranged from 6.5 to 7.0.

As a result of the increasing level of the combination of mBSF-GOE and nBRBROE supplementation in diet, the ruminal NH_3-N content was unchanged after 12 and 24 h of fermentation, and it was not significantly different between treatments due to the higher EO components like CT and SP in both additives. This is consistent with the findings of Makkar [21], who observed that CT or phytogenic oils had nutritional benefits by creating a protein-CT complex or lipid-binding site, thereby decreasing the availability of feed protein for ruminal breakdown and limiting the use of the NH_3-N for ruminal microbial growth. Another finding is in agreement with Viennasay et al. [22], who found that rumen protection in the diet increases when the level of *Flemingia macrophylla* supplementation, containing CT increased, which reduces ruminal NH_3-N concentration. Another possible explanation for this data on NH_3-N content was related to changes in the abundance of species involved in the dynamics of the cellulolytic-ruminal microbial population, particularly *F. succinogenes*, *R. albus*, *R. flavefaciens*, and *M. elsdenii*, while hydrogenation bacteria such as *B. fibrisolvens* and *B. proteocalsticus* did not decrease, similar to the results of Phupaboon et al. [7].

The most striking results of the combined mBSF-GOE and nBRBROE supplementations were the impact on ruminal microbial consortia, in vitro nutrient degradation, and release of the fermentation end-products in terms of VFA profiles, total VFA concentration, and CH_4 production. Especially, the proportion of C_3 and total VFA content which increased with the feed diet in a 4:2 ratio of mBSF-GOE and nBRBROE supplementation, whereas the proportion of C_2 decreased in the same treatment. Consequently, there was a reduction in the anticipated change in VFA profiles from C_2 to C_3, accompanied by a shift in H_2 away from CH_4 production following the methanogenic archaea (e.g., *Methanobacteriales*) population decrease due to antimicrobial activity obtained from the phytogenic oil retained in micro/nano-capsules, which agrees with Patra and Sexena [23] and also Phupaboon et al. [7]. This shift is advantageous for ruminants in terms of their nutritional needs by using phytonutrients to serve as modifiers, following four mechanisms for CT to effectively suppress CH_4 production; (i): *methanogens* are directly affected by these chemicals [24]; (ii): the protozoal group is influenced by CT [25]; (iii): CT serves as a hydrogen sink [26, 27]; and (iv): they have an impact on reducing fiber degradation [28]. Accordingly, Kongmun et al. [17], who recorded that the supplementation of coconut oil and garlic powder in the ratios at 8:4, 4:8, and 0:16 mg of total DM substrate, particularly at 8:4 and 0:16 mg, could decrease CH_4 production and protozoal population (log copy gene numbers) by using qPCR. Additionally, the findings of this study are consistent with those of Molho-Ortiz et al. [15], who evaluated phytochemical-based antimicrobials from EOs from garlic oil, cinnamon oil, and rosemary oil supplemented in vitro gas production techniques. Those supplementations resulted in a decrease in VFA in terms of C_2 and C_2 to C_3 production, microbial mass production, CH_4 production, IVDMD, and total gas production at 24 and 72 h post-fermentation. Furthermore, this corroborates the findings of da Silva et al. [29], who suggested that the microencapsulation and nanoencapsulation techniques supplemented in vitro gas production technique effectively released bioactive compounds derived from plant-phytogenic EOs. These compounds interacted with methanogenic archaea, leading to the mitigation of CH_4 emission through various mechanisms: (I) diffusion, which occurs when the wall structure is intact and is influenced by the physicochemical properties of the wall materials; (II) degradation, which is initiated by the addition of enzymes such as protease and amylase to break down the wall materials; (III) dissolution, which is facilitated by the use of solvents to expand the wall materials and enhance release; (IV) changes in pH and ionic strength, which affect the solubility of the wall materials; and (V) high temperature, which can cause the collapse or melting of the wall materials, promoting release.

Recently, in vitro studies have shown that the encapsulated black soldier fly larvae oil (BSFLO) is a promising feed additive that enhances digestibility and reduces

methane emissions, contributing to sustainable animal nutrition. This study evaluated the effects of encapsulated BSFLO on rumen fermentation, gas production kinetics, methane estimation, and digestibility using an in vitro approach. The results showed that encapsulated BSFLO significantly increased gas production, enhanced rumen digestibility, and reduced methane production. However, it had no significant effect on total ammonia-nitrogen, pH, protozoal population, or total volatile fatty acid values as well as the encapsulated BSFLO supports efficient feeding strategies for improved livestock productivity [6].

Conclusions

In summary, the study utilized microencapsulation and nanoemulsion techniques to protect plant phytogenic essential oils (EOs) made from garlic and riceberry rice bran. These EOs contain bioactive substances such as total phenolic content (TPC) and total flavonoid content (TFC). The study evaluated the effectiveness of these EOs using an in vitro gas technique. The supplementation with mBSF-GOE (955.5 mg GAE/g DM and 94.8 mg QUE/g DM in terms of TPC and TFC) and nRBRBOE (102.3 mg GAE/g RO, 30.2 mg QUE/g RO, and 90.1 total anthocyanin (μg Cy 3-glc/g RO) in the ratio at 4:2 mg of the diet demonstrated superior efficiency compared to other treatments in terms of gas production kinetics, cumulative gas production, in vitro dry matter digestibility (IVDMD), proportion of propionate, total volatile fatty acid (VFA) concentration, and reduction of the methanogen population, leading to decreased methane (CH_4) production. Therefore, according to this study, these supplementation mixtures could serve as alternative feed enhancers to enhance ruminal fluid fermentation and end-products. This includes underline the effectiveness of "manipulating" methanogens and the relative decrease in CH_4 production without affecting other parameters (total VFA production or the relative abundance of ruminal microbiome species).

Materials and methods

Animal ethics approval

The ethics committee of Khon Kaen University, Thailand, granted approval for all procedures related to the maintenance, feeding, and collection of rumen fluid from the animals. This approval was obtained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of Khon Kaen University and the Institute of Animals for Scientific Purpose Development (IAD) in Thailand. The relevant records for these procedures are IACUC-KKU-110/66, IACUC-KKU-86/66, and U1-10937-2566. In addition, our study confirmed that all methods were performed in accordance with the relevant guidelines and regulations in compliance with the ARRIVE guidelines.

Essential oils, microencapsulation/nanoemulsion process, and phytonutrients analysis

The essential oils of garlic and riceberry rice bran extracts, using a cool extraction technique, were used as plant phytogenic-based bioactive components through the formulation by encapsulating and emulsifiying techniques and purchased from a local market in Khon Kaen province, Thailand.

The microencapsulation process was modified from the procedure used by Phupaboon et al. [10]. A commercial black soldier fly (BSF)-based protein extract was used as an encapsulant or wall material and formulated with garlic oil extract (GOE) at 1:1 ratio consisting of 20% (w/v) BSF-based protein extract suspension mixed with 10% (v/v) GOE containing 1% (v/v) Tween 80 solution. Afterward, homogeneously media processed through the spray-drying technique at the processing conditions; operation speed (10 mL/min), drying airflow (600 L/h), pressure drop (0.75 bar), inlet temp. (160 °C), and outlet temp. (90 °C). After spray drying, the products of mBSF-GOE powders were kept in a vacuum bag and stored at -20 °C until use in the in vitro gas fermentation.

Subsequently, the nanoemulsion process was prepared according to the procedure of oil-in-water nanoemulsion used by El-Sherbiny et al. [14], with some modifications. Briefly, riceberry rice bran oil extract (RBRBOE) was premixed at 1500 rpm for 1 min with DI water at a 15:79.4% (v/v) ratio using a digital high-speed HG-15D homogenizer (Daihan Scientific, South Korea) for homogeneous dissolving and a smaller droplet size. Following that, the homogeneous media of an oil-in-water emulsion was remixed with 5.6% (v/v) of Tween 80 at the same conditions. Next step, nanoemulsion particles were formulated by utilizing an ultrasonic bath-precision GP 10 (Thermo Scientific, USA) with a nominal power of 750 W at room temperature for 20 min and a frequency of 20 kHz. Later, the product of the nanoemulsified RBRBOE (nRBRBOE) solution was kept at 4 °C and directly weighed into in vitro gas bottles.

Table 1 shows the formulation of the dietary treatment concentrate, which was composed of a combination of ingredients and mineral premix. The dried materials underwent chemical analysis using the approach outlined by AOAC [30] protocol to determine their chemical compositions: dry matter (DM; no. 967.03), ash (no. 942.05), CP (no. 948.13). The fiber fractions in terms of the neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed by using Ankom fiber analyzer (Ankom Technology, USA) according to the procedure of Van Soest et al. [31]. Furthermore, the bioactive components of the additives mBSF-GOE and nRBRBOE were examined using a modification of the methodology outlined by Phupaboon et al. [10] in terms of total polyphenolic content (TPC), total flavonoid content (TFC),

and antioxidative values: 2,2-diphenyl-1-picrylhydrazyl (DPPH assay), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS assay), radical scavenging inhibition, and ferric reducing antioxidant power (FRAP assay), as shown in Table 1. Moreover, the calculation of encapsulation efficacy (EE) was described by Phupaboon et al. [7] using the equation: EE (%) = (Amount of TPC extracted)/(Amount of TPC entrapped) x 100.

Experimental design and treatment details

The experimental design in this study was a completely randomized design (CRD). Subsequently, the treatment details were supplemental mixing with mBSF-GOE and nRBRBOE at varying ratios of 0:0, 6:0, 4:2, 2:4, and 0:6 mg in 500 mg of diets, respectively. Rice straw as a roughage to concentrate (R: C) ratio at 60:40 was used as a diet substrate.

The details of this study's experimental design (ED) were separated to investigate the different parameters consisting of (ED-I): determination of gas production parameter (total of 15 bottles from 3 bottles/treatments, for 96 h of incubation times); (ED-II): analysis of nutrient degradability and CH_4 production parameters (total of 20 bottles from 2 bottles/treatments, for assay at 12 and 24 h); and (ED-III): analysis of VFA and NH_3-N concentrations along with microbiota population parameters (total of 20 bottles from 2 bottles/treatments, for assay at 12 and 24 h) according to the procedure of Phupaboon et al. [7].

Donor rumen fluid collection, preparation, and in vitro fermentation trials

The animals, utilized as a rumen supply, were sourced from the Faculty of Animal Science farm at Khon Kaen University, Khon Kaen, Thailand. The collection took place 3 h after the morning feeding following the protocol of National Research Council (NRC) [32], with fed a diet of the same composition as the basal diet and water *ad libitum*. Approximately 1200 mL of total rumen fluid were obtained from three donor Thai native cattle by using suction pump equipment, and they had an average body weight of 300 ± 10 kg. The rumen fluid that was gathered was passed through four layers of cloth sheets and deposited into a preheated thermos flask at a temperature of 39 °C. The flask was promptly transported to the laboratory within a time frame of 15 min.

Part of the rumen inoculum medium was prepared by mixing together with the filtered rumen fluid sample and the generated artificial saliva solution (containing micro-mineral solution, resazurine, reduction solution, and macro-mineral solution) in a 2:1 (v/v) ratio, and then incubated at a temperature of 39 °C while being continuously flushed with CO_2 [33].

To begin in vitro gas fermentation, 500 mg of all the dietary substrates were prepared by weighing the R: C ratio at 60:40 added into 60 mL in vitro glass bottles and adding both of the additives (mBSF-GOE and nRBRBOE) in the order specified in the treatment details (at ratios 0:0, 6:0, 4:2, 2:4, and 0:6 mg in diet). Then, all bottles were hermetically sealed with rubber stopper and aluminium caps. Subsequently, 40 mL of rumen inoculum medium were added and continuously flushed with CO_2 to maintain anaerobic conditions and then incubated at 39°C[7].

Sample collection and their chemical analysis

Following ED-I, -II, and -III of post-fermentation, the total gas production in the headspace of the glass bottles was directly measured by syringe with a needle obtained from ED-I. Under this experiment, kinetic gas production during incubation was measured at 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h, and the data was fitted to the cumulative gas production model of Ørskov and McDonald [34] as follows: $Y = a + b\ (1 - e^{-ct})$, where Y = gas produced at time "t" (mL), a = the gas production from the immediately soluble fraction (mL), b = the gas production from the insoluble fraction (mL), and c = the gas production rate constant for the insoluble fraction (mL/h).

Based on the ED-II after 12 and 24 h of post-incubation, to characterize the in vitro rumen fermentation and to establish the in vitro DM degradability (IVDMD; % DM) as following Van Soest et al. [31], directly ruminal pH and content of CH_4 production using a GC instrument (GC-2014, Shimazu Co., Kyoto, Japan), it was calculated as following the equation: CH_4 production = (peak area/slope)/(volume of 0.3 mL of gas sampling collected from the headspace of the glass bottles) [7]. Other the ED-III of the rumen fluid samples at 12 and 24 h-post fermentation was separated into two portions; the first supernatant portion containing 10% (w/v) H_2SO_4 was previously provided insoluble fiber by filtration followed by centrifugation and kept at -20 °C for analysis of the VFA (mol/100 mL) profiles utilizing a GC instrument (Nexis GC-2030 equipped with SH-Rt Q-BOND Column, 30 m, 0.53-mm ID, 20 µm, Shimadzu Co., Kyoto, Japan) [7] and NH_3-N (mg/dL) content using an NH_3-N kit (FUJIFILM Wako Pure Chemical Corp, Japan) [35]. Additionally, the second supernatant portion was mixed with a smaller insoluble fiber fraction and used for DNA extraction according to the procedure of Phupaboon et al. [36] by using the GF-1 bacterial DNA extraction kit (Vivantis, Malaysia) to determine the rumen microbiota using the real-time PCR (RT-PCR) technique. The reaction of the RT-PCR assay using the Chromo 4™ system (Bio-Rad, USA) was conducted with purified DNA (OD260/280 = 1.8 to 2.0) as a DNA template at a final quantity of 0.05 µg/µL mixed together with SYBR green master mix reagent (Luna® Universal qPCR Master Mix,

New England Biolabs GmbH, Germany), the specify Fw and Rw primers, and adjusted to a final volume of 10 µL with free-Ranse water, followed by the optimal annealing condition at 55 ℃ [37]. The species-specific PCR primer used to determine the rumen microbial population includes the species *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* [38], *Megasphaera elsdenii* [39], *Butyribrio fibrisolvens* [40], and *Methanobacteriales* [41]. The data of the microbial population was counted in duplicate from each sample to calibrate with the absolute abundance group of each specific species and expressed as log 10 gene copies per mL of DNA template obtained from rumen fluid samples [42].

Statistical analysis

The collected data were analyzed using the General Linear Model (GLM) approaches of SAS Software version 9.0 (SAS Inst. Inc., Cary, NC, USA) run on a completely randomized design (CRD) platform by model: $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where Y_{ij} = the observation, μ = the overall mean, α_i = the treatment effect, and ε_{ij} = the residual error. We used Duncan's New Multiple Range Test (DMRT) to compare the mean values of the experimental treatments. Treatment means were considered significantly different if their *P*-values were < 0.05 and < 0.01, respectively. The reactions to mBSF-GOE and nRBRBOE supplementation were evaluated using orthogonal polynomials to identify trends.

Abbreviations

%EE	Percentage of encapsulation efficiency
ABTS	2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ADF	Acid detergent fiber
CH_4	Methane
CO_2	Carbon dioxide
CTs	Condensed tannins
DM	Dry matter
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EOs	Essential oils
FRAP	Ferric reducing antioxidant power
Fw	Forword primer
GHG	Greenhouse gas
GOE	Garlic oil extract
H_2	Hydrogen
IVDMD	In vitro dry matter degradability
mBSF-GOE	Microencapsulated black soldier fly with garlic oil extract
NDF	Neural detergent fiber
NH_3-N	Ammonium nitrogen
nRBRBOE	Nanomulsified riceberry rice bran oil extract
qPCR	Qualitative polymerase chain reaction
RBRBOE	Riceberry rice bran oil extract
RT-PCR	Real time-polymerase chain reaction
Rw	Reword primer
SPs	Saponins
TFC	Total flavonoid content
TPC	Total polyphenolic content
VFAs	Volatile fatty acid

Acknowledgements

The authors are thankful to Dr. Peter Rowlinson, an esteemed independent animal science consultant, for his invaluable suggestions to enhance the manuscript. Additionally, the authors would like to acknowledge the Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand, for access to the facilities.

Author contributions

S.P.: Project administration, Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Software, Validation, Visualization; U.M., C.S., S.S., M.M., G.D., and R.P.: Methodology, Conceptualization, Writing– original draft (read); M.W.: Funding acquisition, Resources, Supervision, Conceptualization; S.P. and M.W.: Writing– original draft, Writing– review & editing. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

Most sincere thanks are extended to the Fundamental Found (FF-68) (grant number 68A103000138) under Khon Kaen University, which has received funding support from the National Science Research and Innovation Fund (NSRF), Thailand.

Data availability

The datasets generated and analyzed during the present study are available within the article.

Declarations

Ethics approval and consent to participate

The authors confirmed that rumen sources obtained informed consent from the Faculty of Animal Science farm at Khon Kaen University, Khon Kaen, Thailand. Also, 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 Animals for Scientific Purpose Development (IAD) in Thailand. The pertinent records for these procedures include IACUC-KKU-110/66, IACUC-KKU-86/66, and U1-10937-2566. Furthermore, our study verified that all methods adhered to the pertinent guidelines and regulations, in alignment with the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 7 April 2025 / Accepted: 6 May 2025
Published online: 17 May 2025

References

1. O'Mara FP. The significance of livestock as a contributor to global greenhouse gas emissions today and in the near future. Anim Feed Sci Technol. 2011;166–167:7–15.
2. Wanapat M, et al. Effects of plants containing secondary compounds and plant oils on rumen fermentation and ecology. Trop Anim Health Prod. 2012;44:399–405.
3. Goncagul G, Ayaz E. Antimicrobial effect of garlic (*Allium sativum*). Recent Pat Anti-Cancer Drug Discov. 2010;5:91–3.
4. Ee KY, et al. Optimizing protein hydrolysates of rice bran: physicochemical, antioxidant, antibacterial properties, and chemometric analysis for functional food potential. Meas: Food. 2024;13:100141.
5. Manlapig JJD, et al. Effect of rice bran extract on in vitro rumen fermentation and methane production. Anim Sci J. 2024;95:e13923.
6. Albarki HR, et al. Sustainable animal nutrition and feeding strategies for reducing methane emissions and enhancing feed digestibility with encapsulated black soldier fly larvae oil. Sustainability. 2025;17:2071–1050.
7. Phupaboon S, et al. Microencapsulation efficiency of fruit peel phytonutrient-based antimicrobial to mitigate rumen emission using in vitro fermentation technique. Ital J Anim Sci. 2024;23:664–77.

8. Bento MHL, et al. Essential oils and their use in animal feeds for monogastric animals-effects on feed quality, gut microbiota, growth performance and food safety: a review. Vet Med. 2013;58:449–58.
9. Matra M, et al. Microencapsulation of *Mitragyna* leaf extracts to be used as a bioactive compound source to enhance in vitro fermentation characteristics and microbial dynamics. Anim Biosci. 2024;37:74.
10. Phupaboon S, et al. Extraction, characterization, and chitosan microencapsulation of bioactive compounds from Cannabis sativa L., Cannabis indica L., and Mitragyna speiosa K. Antioxidants. 2022;11:2103.
11. Amin N, Tagliapietra F, Arango S, Guzzo N, Bailoni L. Free and microencapsulated essential oils incubated in vitro: ruminal stability and fermentation parameters. Animals. 2021;11:180.
12. Kim TB, Lee JS, Cho SY, Lee HG. In vitro and in vivo studies of rumen-protected microencapsulated supplement comprising linseed oil, vitamin E, rosemary extract, and hydrogenated palm oil on rumen fermentation, physiological profile, milk yield, and milk composition in dairy cows. Animals. 2020;10:1631.
13. Muslykhah U, Phupaboon S, Suriyapha C, Matra M, Wanapat M. Encapsulation of protein-based bioactive from black soldier fly for ruminant feeding. J Agric Food Res. 2024a;18:101325.
14. El-Sherbiny M, et al. Oil-in-water nanoemulsion can modulate the fermentation, fatty acid accumulation, and the microbial population in rumen batch cultures. Molecules. 2023;28:358.
15. Molho-Ortiz AA, et al. Effect of essential oils and aqueous extracts of plants on in vitro rumen fermentation and methane production. Anim Behav Biometeorol. 2021;10:2110.
16. Griffin SG, Wyllie SG, Markham JL, Leach DN. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour Fragr J. 1999;14:322–32.
17. Kongmun P, Wanapat M, Pakdee P, Navanukraw C. Effect of coconut oil and garlic powder on in vitro fermentation using gas production technique. Livest Sci. 2010;127:38–44.
18. Pal K, Patra AK, Sahoo A. Evaluation of feeds from tropical origin for in vitro methane production potential and rumen fermentation in vitro. Span J Agric Res. 2015;13:0608.
19. Phesatcha K, Phesatcha B, Wanapat M, Cherdthong A. Roughage to concentrate ratio and *Saccharomyces cerevisiae* inclusion could modulate feed digestion and in vitro ruminal fermentation. Vet Sci. 2020;7:151.
20. Wanapat M. Manipulation of cassava cultivation and utilization to improve protein to energy biomass for livestock feeding in the tropics. Asian-Australas J Anim Sci. 2003;16:463–72.
21. Makkar HP. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin Res. 2003;49:241–56.
22. Viennasay B, et al. Effect of *Flemingia macrophylla* silage on in vitro fermentation characteristics and reduced methane production. Anim Prod Sci. 2020;60:1918.
23. Patra AK, Saxena J. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J Sci Food Agric. 2011;91:24–37.
24. Carrasco JMD, et al. Impact of chestnut and Quebracho tannins on rumen microbiota of bovines. BioMed Res Int. 2017;9610810:11.
25. Bhatta R, et al. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J Dairy Sci. 2009;92:5512–22.
26. Becker PM, et al. Evidence for a hydrogen-sink mechanism of (+) catechin-mediated emission reduction of the ruminant greenhouse gas methane. Metabolomics. 2014;10:179–89.
27. Muslykhah U, et al. Potential use of *Wolffia globosa* powder supplementation on in vitro rumen fermentation characteristics, nutrient degradability, microbial population, and methane mitigation. Sci Rep. 2024b;14:28611.
28. Carulla JE, Kreuzer M, Machmüller A, Hess HD. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Austr J Agric Res. 2005;56:961–70.
29. da Silva PT, et al. Microencapsulation: concepts, mechanisms, methods and some applications in food technology. Cienc Rural. 2014;44:1304–11.
30. AOAC. Official methods of analysis. 19th ed. USA: Association of Official Analytical Chemists, VA; 2012.
31. Van Soest PV, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 1991;74:3583–97.
32. National Research Council. The committee on animal nutrition, & subcommittee on dairy cattle nutrition, nutrient requirements of dairy cattle: 2001. National Academies; 2001.
33. Menke KH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Anim Res Dev. 1988;28:7–55.
34. Ørskov ER, McDonald I. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J Agric Sci. 1979;92:499–503.
35. Ahmed E, Fukuma N, Hanada M, Nishida T. The efficacy of plant-based bioactives supplementation to different proportion of concentrate diets on methane production and rumen fermentation characteristics in vitro. Animals. 2021;11:1029.
36. Phupaboon S, Hashim FJ, Phumkhachorn P, Rattanachaikunsopon P. Molecular and biotechnological characteristics of proteolytic activity from *Streptococcus thermophilus* as a proteolytic lactic acid bacteria to enhance protein-derived bioactive peptides. AIMS Microbiol. 2023;9:591–611.
37. Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J Biotechnol. 2006;123:273–80.
38. Koike S, Kobayashi Y. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes, Ruminococcus albus and Ruminococcus flavefaciens*. FEMS Microbiol Lett. 2001;204:361–6.
39. Ouwerkerk D, Klieve AV, Forster RJ. Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. J Appl Microbiol. 2002;92:753–8.
40. Fernando SC, et al. Rumen microbial population dynamics during adaptation to a high-grain diet. Appl Environ Microbiol. 2010;76:7482–90.
41. Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng. 2005;89:670–9.
42. Whelan JA, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods. 2003;278:261–9.

Publisher's note