

Article

The Efficacy of Plant-Based Bioactives Supplementation to Different Proportion of Concentrate Diets on Methane Production and Rumen Fermentation Characteristics In Vitro

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Simple Summary: Using natural feed additives to mitigate methane emissions from ruminants is a promising strategy. Many antimethanogenic compounds have been used to alter rumen fermentation, yet their potential to reduce methane production effectively is not consistent across different kinds of feeding styles (forage:concentrate ratios). Consequently, in the current study we investigated the efficacy of plant-bioactives extract (PE) (a novel phytogetic mixture of garlic and citrus extracts) on rumen fermentation characteristics and methane production in different kinds of feeding styles. The current In Vitro study showed that PE was effective in reducing methane production in all feeding styles without exhibiting any adverse effect on nutrient digestibility. Furthermore, PE supplementation was able to improve the rumen fermentation through increasing the production of total volatile fatty acids. Therefore, PE mixture could be used as a dietary supplement to reduce the methane production from ruminants.

Abstract: This In Vitro study was conducted to investigate the impact of plant-bioactives extract (PE), a combination of garlic powder and bitter orange extract, on methane production, rumen fermentation, and digestibility in different feeding models. The dietary treatments were 1000 g grass/kg ration + 0 g concentrate/kg ration (100:0), 80:20, 60:40, 40:60, and 20:80. The PE was supplemented at 200 g/kg of the feed. Each group consisted of 6 replicates. The experiment was performed as an In Vitro batch culture for 24 h at 39 °C. This procedure was repeated in three consecutive runs. The results of this experiment showed that supplementation with PE strongly reduced methane production in all kinds of feeding models ($p < 0.001$). Its efficacy in reducing methane/digestible dry matter was 44% in the 100:0 diet, and this reduction power increased up to a 69.2% with the inclusion of concentrate in the 20:80 diet. The PE application significantly increased gas and carbon dioxide production and the concentration of ammonia-nitrogen, but decreased the pH ($p < 0.001$). In contrast, it did not interfere with organic matter and fiber digestibility. Supplementation with PE was effective in altering rumen fermentation toward less acetate and more propionate and butyrate ($p < 0.001$). Additionally, it improved the production of total volatile fatty acids in all feeding models ($p < 0.001$). In conclusion, the PE combination showed effective methane reduction by improving rumen fermentation characteristics without exhibiting adverse effects on fiber digestibility. Thus, PE could be used with all kinds of feeding models to effectively mitigate methane emissions from ruminants.

Keywords: garlic; citrus; methane emission; rumen fermentation; digestibility



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1. Introduction

Due to the continuous expansion of the world population, human demand for meat and milk is expected to increase by 73% and 58%, respectively, by 2050 compared with 2010 levels [1]. Therefore, to meet future needs, animal production must be increased. Although the livestock sector, especially ruminants, plays an essential role in food security, it is considered a significant source of greenhouse gases (GHGs), such as methane (CH₄) and carbon dioxide (CO₂), representing approximately 14–18% of global anthropogenic GHG emissions depending on the accounting approaches by various sources (IPCC, FAO, or others) [2]. These GHGs are directly related to global warming and climate change, which threaten the well-being of current and future generations [3]. Ruminants emit CH₄ as a byproduct of their normal digestive process due to fermentation of feed. The CH₄ released from enteric fermentation through eructation represents a loss of up to 15% of their gross energy intake, thus being one of the most important inefficiencies in ruminant production systems in addition to its environmental impact, since CH₄ is 28 times more powerful than CO₂ at trapping the sun's heat [2,4].

Currently, studies are being performed of abatement strategies to reduce CH₄ emissions and to improve the performance of ruminants. Through these studies, it has been proven that manipulation of the rumen microbiome with dietary supplements is one of the best mitigation strategies and that it has a two-sided benefit for both the environment and efficient livestock production [5,6]. Several research groups worldwide are investigating different kinds of feed additives/supplements with antimicrobial activity; however, the results reported in the literature are variable and show inconsistent efficacy [7,8]. One of the major reasons for the inconsistent effectiveness is related to the type of diet: forage- or concentrate-based diets [9]. It is often assumed that diet composition has a role in CH₄ formation [10,11]. Therefore, there is an urgent need for a feed supplement that could achieve effective reduction of emissions with different kinds of ruminant diets without impairing rumen fermentation; meanwhile, supplements should be natural, as there is global interest in using plants and their secondary metabolites as alternatives to chemical compounds/antimicrobials in animal feed, and natural supplements are acceptable to consumers [12]. Nowadays, the potential of using phytochemicals gives the possibility of decreasing the negative impact of animal production on environment, but still, more effective supplements are required [6].

Plant-bioactives extract (PE), a mixture of organosulfur compounds extracted from garlic (*Allium sativum*) and flavonoids extracted from bitter orange (*Citrus aurantium*), showed the ability to reduce CH₄ production without impairing rumen fermentation characteristics in two In Vitro studies [13,14]. We hypothesized that PE supplementation might have the same mode of action and the potential to reduce CH₄ regardless of the grass:concentrate ratio. However, there are still limitations to proving the efficacy of this new combination with different feeding models (grass:concentrate ratios). Therefore, this study was conducted to investigate the potential of PE to be used as an antimethanogenic feed supplement with different feeding styles of ruminants, considering its impact on rumen fermentation and nutrient digestibility.

2. Materials and Methods

2.1. Donor Animals and Rumen Fluid Collection

The animals used in this experiment were kept and cared for by the Field Science Center, Obihiro University of Agriculture and Veterinary Medicine, Japan. The animal management and sampling procedures were approved by the Obihiro University of Agriculture and Veterinary Medicine, Animal Care and Use Committee (Approval number, 20-119).

At 3 h after the morning feeding, approximately 3 L of rumen fluid was collected from two ruminally fistulated, nonlactating Holstein cows (880 kg average body weight). The cows were maintained on a daily diet of Orchard grass (*Dactylis glomerata*) hay (organic matter (OM), 980 g/kg; crude protein (CP), 132 g/kg; neutral detergent fiber (NDF),

701 g/kg; acid detergent fiber (ADF), 354 g/kg; acid detergent lignin (ADL), 40 g/kg; on dry matter (DM) basis) with free access to clean drinking water and mineral blocks (KOEN® E250 TZ, Nippon Zenyaku Kogyo Co., Fukushima, Japan). The rumen fluid from each cow was collected from four different locations in the rumen. The collected rumen fluid was strained through four layers of surgical gauze into a thermos flask that was prewarmed to 39 °C and then immediately transferred to the laboratory within 15 min.

2.2. Experimental Treatments and In Vitro Incubation Technique

Prior to the In Vitro incubation, ten experimental groups, with six replicates each, were prepared with approximately 500 mg (fresh matter) of ground substrate composed of Kleingrass (*Panicum coloratum*) hay and commercial concentrate mixture at different ratios with and without PE inclusion. The PE mixture was composed of 90% garlic granules (Allicin) and 10% citrus extract powder (Naringin, Naringenin, Neohesperidin, Rhoifolin, and Neoeriocitrin). The garlic powder used for PE preparation was sourced from cultivated and carefully processed and dried non-GMO garlic of Chinese origin. The dried garlic granules were standardized to contain 1% (w/w) allicin potential (S-Prop-2-en-1-yl prop-2-ene-1-sulfinothioate). Allicin concentration was determined by high performance liquid chromatography (HPLC) as described in details by Eger et al. [13]. The citrus components for the PE mixture (Naringin, Naringenin, Neohesperidin, Rhoifolin, and Neoeriocitrin) were developed from commercially available citrus extracts (Khush Ingredients, Oxford, United Kingdom) mainly extracted from bitter oranges (*Citrus aurantium*). The total polyphenol content of the citrus extract was standardized to 45% (w/w) by the Folin—Ciocalteu method [15]. Flavonoid concentrations were analyzed by HPLC using standards from (Sigma-Aldrich Ltd., Dorset, UK). Further information on the PE preparation was described in detail by Eger et al. [13]. This PE mixture is known commercially as Mootral and was provided by a Swiss company (Mootral SA, Rolle, Switzerland).

The experimental diets were as follows: 1- 1000 g grass/kg ration + 0 g concentrate/kg ration (100:0), 2- 100:0 + 200 g PE/kg of substrate (20 PE), 3- 80:20, 4- 80:20 + 20 PE, 5- 60:40, 6- 60:40 + 20 PE, 7- 40:60, 8- 40:60 + 20 PE, 9- 20:80, and 10- 20:80 + 20 PE. Five hundred milligrams of each of the experimental substrates (grass and concentrate) was added to preweighed ANKOM filter bags (F57, ANKOM Technology, Macedon, NY, USA), which were heat-sealed and placed in 120 mL glass bottles, whereas the PE mixture was added directly to the bottles (out of filter bag) one day before incubation. The dosage of PE used in the current study was based on the effective dose that altered the bacterial and archaeal communities in our previous In Vitro study [14]. Therefore, that effective dosage had to be used in further In Vitro trials for better understanding the mode of action of this new mixture. The chemical composition of the substrates and the PE are described in Table 1.

Table 1. Chemical composition of ration and plant-bioactives extract (g/kg of dry matter) used in 24 h In Vitro incubation.

(g/kg Dry Matter)	Kleingrass Hay	Concentrate	Plant-Bioactives Extract
Dry matter (g/kg fresh matter)	844.9	843.0	871.5
Organic matter	904.7	934.2	955.5
Crude ash	95.3	65.8	44.5
Crude protein	134.8	223.1	210.7
Ether extract	38.4	38.0	17.1
Neutral detergent fiber	662.5	232.6	35.5
Acid detergent fiber	362.6	109.1	33.7
Acid detergent lignin	52.2	30.1	1.8

The procedure of In Vitro batch culture was performed as described by Menke and Steingass [16]. In the laboratory, the collected rumen fluids from the two cows were mixed together in one beaker under a constant stream of CO₂. Forty milliliters of fresh buffer

solution at a pH of 6.8 prepared according to McDougall [17] with twenty mL of rumen fluid was added to each 120 mL bottle under continuous CO₂ flushing to maintain anaerobic conditions. Thereafter, the fermentation bottles were flushed with CO₂ before sealing with butyl rubber stoppers and aluminum caps (Maruemu Co., Ltd, Osaka, Japan). All bottles were incubated for 24 h at 39 °C. This batch culture procedure was repeated in three consecutive runs on three different days. In each run, two blanks without substrate (empty filter bag plus 60 mL of buffered rumen fluid) were included to be used for digestibility and gas production correction. In total, 180 bottles plus 6 blank bottles were examined in this study.

2.3. Sample Collection

After 24 h of incubation, the total gas production was measured, and gas samples were collected from the headspace of the glass bottles into vacutainer tubes (BD Vacutainer[®], Becton Drive, Franklin Lakes, NJ, USA). The tubes were stored at room temperature until CH₄ and CO₂ determination. Thereafter, the bottles' caps were removed, and the pH of each tube was recorded using a pH meter (LAQUA F-72, HORIBA Scientific, Kyoto, Japan). Then, aliquots of the culture fluid were transferred into 1.5 mL Eppendorf tubes and centrifuged at 16,000 × *g* and 4 °C for 5 min. The supernatant was collected and transferred into a new Eppendorf tube[®] (Eppendorf AG, Hamburg, Germany), which was stored at −20 °C until use for volatile fatty acid (VFA) and ammonia nitrogen (NH₃-N) analysis. The bags were removed from the bottles, washed under running tap water until the draining fluid became clear, and then dried at 60 °C for 48 h to determine the In Vitro dry matter digestibility (IVDMD). After IVDMD determination, the bags were used for the determination of In Vitro organic matter digestibility (IVOMD), In Vitro neutral detergent fiber digestibility (IVNDFD), and In Vitro acid detergent fiber digestibility (IVADFD). The residues in the fermentation bottles were discarded.

2.4. Chemical Analysis

The chemical composition of the grass, concentrate, PE, and remaining substrate in the bags was determined following the standard procedure of AOAC [18]. The DM content was measured by drying the samples in an air-forced oven at 135 °C for 2 h (930.15). OM and ash were measured by placing the samples into a muffle furnace at 500 °C for 3 h (942.05). Nitrogen (N) was measured according to the method of Kjeldahl (984.13) using an electrical heating digester (DK 20, VELP Scientifica, Usmate (MB), Monza, Italy) and an automatic distillation apparatus (UDK 129 VELP Scientifica, Usmate (MB), Monza, Italy), and then CP was estimated as N × 6.25. The ADF, NDF, and ADL were measured and expressed as inclusive residual ash using an ANKOM²⁰⁰ Fiber Analyzer (Ankom Technology Methods 5, 6, and 8, respectively; ANKOM Technology Corp., Macedon, NY, USA). The NDF was measured using sodium sulfite with heat-stable α-amylase.

2.5. Gas Composition Analysis

The concentrations of CH₄ and CO₂ in the gas samples were determined by injection of 1 mL using a Hamilton gastight syringe (Hamilton Company, Reno, NV, USA) into a gas chromatograph (GC-8A, Shimadzu Corp., Kyoto, Japan). The carrier gas was helium. The temperatures of the infuser port, column, and detector were 70 °C, 150 °C, and 150 °C, respectively. The identification of CH₄ and CO₂ was based on the retention time.

2.6. Volatile Fatty Acids and Ammonia-Nitrogen Analysis

The concentration of VFA was determined using high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) after diluting the supernatant 3 times with distilled water. Briefly, the analytical specifications were as follows: column, Shim-pak SCR-102H (7 mm, i.d. 8.0 mm × 300 mm, Shimadzu Corp., Kyoto, Japan); eluent flow rate and mobile phase for organic acid analysis (Shimadzu Corp., Kyoto, Japan) at 0.8 mL/min; column temperature, 40 °C; reaction reagent and flow rate, pH buffer for organic acid analysis (Shi-

madzu Corp., Kyoto, Japan) at 0.8 mL/min; conductivity detector (CDD-10AVP, Shimadzu Corp., Kyoto, Japan). Quantification of the VFA concentration was performed using an external standard quantitation method [14].

The NH₃-N concentration was measured by diluting samples 50 times with 0.1 M phosphate buffer (pH 5.5) and then they were analyzed following the procedure of the modified Fujii—Okuda method [19] using an NH₃ kit (FUJIFILM Wako Pure Chemical Corp, Osaka, Japan). The plate was read by a microplate reader (SH-1000 Lab, Corona Electric Co., Ltd., Japan) at an optical density of 630 nm.

2.7. Statistical Analysis

All variables were analyzed using PROC MIXED by SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The model included the treatment (diet) effect, PE effect, and their interaction as fixed effects, whereas the experimental runs were considered random effects. Least square means and standard error (SEM) were calculated, and the differences of means were estimated by pairwise *t*-tests (PDIF option of PROC MIXED). Significance was declared at $p < 0.05$, and a tendency toward significance was declared when the p value was between 0.05 and 0.10.

3. Results

3.1. Effect of Plant-Bioactives Extract Supplementation on In Vitro pH, Gas Production, and Gas Composition

Supplementation of PE to all feeding models reduced the pH ($p < 0.001$) when compared with its corresponding group without PE supplementation in the same feeding model (Table 2). Moreover, the inclusion of PE increased the absolute total gas production when correlated with DM and digestible DM in all feeding styles ($p < 0.001$, Table 2).

Table 2. Effect of plant-bioactives extract (PE) supplementation on gas production and pH in different feeding styles after 24 h In Vitro incubation ($n = 18$).

Parameter	Treatments ¹										SEM	<i>p</i> -Value		
	100:0		80:20		60:40		40:60		20:80			Trt	PE	Trt × PE
	0	20	0	20	0	20	0	20	0	20				
Gas production (mL)	30.06	45.53 ***	40.18	51.19 ***	43.50	53.08 ***	44.17	57.14 ***	45.94	54.47 ***	0.74	<0.001	<0.001	0.07
Gas/DM ² (mL/g)	71.17	107.67 ***	94.90	120.93 ***	102.89	125.44 ***	104.31	135.09 ***	108.68	128.87 ***	1.76	<0.001	<0.001	0.07
Gas/Digestible DM (mL/g)	191.13	281.87 ***	207.46	288.09 ***	209.23	247.66 ***	200.41	266.66 ***	197.98	241.21 ***	3.18	<0.001	<0.001	<0.001
pH	6.58	6.50 ***	6.55	6.47 ***	6.53	6.44 ***	6.51	6.41 ***	6.50	6.40 ***	0.01	<0.001	<0.001	0.36

¹ grass:concentrate ratio; 0: 0 g PE/kg; 20: 200 g PE/kg of substrate. ² DM: dry matter. Asterisks in 20 mean significant difference between 0 and 200 g PE/kg in the same feeding model, *** ($p < 0.001$). Trt: treatment; PE: plant-bioactives extract; Trt × PE: interaction between treatment and plant-bioactives extract. SEM: standard error of the mean.

Adding PE to all feeding styles decreased the proportion of CH₄ but increased the proportion of CO₂ in the produced gas ($p < 0.001$, Table 3). Furthermore, the CH₄/CO₂ ratio in the produced gas (mL/mL) decreased in all feeding models due to PE's effect ($p < 0.001$, Table 3). The PE inclusion was effective with all diets in reducing the production of CH₄/DM (mL/g) ($p < 0.001$); moreover, it reduced the production of CH₄/digestible DM (mL/g) by 44.2%, 48.2%, 59.7%, 63.7%, and 69.2% in 100:0, 80:20, 60:40, 40:60, and 20:80, respectively, ($p < 0.001$). In contrast, the production of CO₂/DM and CO₂/digestible DM (mL/g) increased ($p < 0.001$) due to the effect of PE in all feeding styles (Table 3).

Table 3. Effect of plant-bioactives extract (PE) supplementation on CH₄ and CO₂ production in different feeding styles after 24 h In Vitro incubation ($n = 18$).

Parameter	Treatments ¹										SEM	p-Value		
	100:0		80:20		60:40		40:60		20:80			Trt	PE	Trt × PE
	0	20	0	20	0	20	0	20	0	20				
CH ₄ (%)	5.19	1.99 ***	5.59	2.07 ***	5.87	1.84 ***	5.83	1.59 ***	5.89	1.51 ***	0.16	0.006	<0.001	<0.001
CO ₂ (%)	94.81	98.01 ***	94.41	97.93 ***	94.13	98.16 ***	94.17	98.41 ***	94.11	98.49 ***	0.16	0.006	<0.001	0.21
CH ₄ /CO ₂ ratio (mL/mL)	0.055	0.020 ***	0.059	0.021 ***	0.062	0.019 ***	0.062	0.016 ***	0.063	0.015 ***	0.002	0.004	<0.001	<0.001
CH ₄ /DM ² (mL/g)	3.68	2.09 ***	5.36	2.57 ***	6.11	2.30 ***	6.09	2.16 ***	6.48	1.91 ***	0.16	<0.001	<0.001	<0.001
CO ₂ /DM (mL/g)	67.45	105.58 ***	89.50	118.36 ***	96.78	123.14 ***	98.22	132.93 ***	102.20	126.40 ***	1.78	<0.001	<0.001	0.12
CH ₄ /digestible DM (mL/g)	9.88	5.51 ***	11.62	6.02 ***	12.35	4.98 ***	11.64	4.23 ***	11.66	3.59 ***	0.29	<0.001	<0.001	<0.001
CO ₂ /digestible DM (mL/g)	181.26	276.36 ***	195.85	282.07 ***	196.89	269.68 ***	188.77	262.43 ***	186.33	237.60 ***	3.35	<0.001	<0.001	<0.001

¹ grass:concentrate ratio; 0: 0 g PE/kg; 20: 200 g PE/kg of substrate. ² DM: dry matter. Asterisks in 20 mean significant difference between 0 and 200 g PE/kg in the same feeding model, *** ($p < 0.001$). Trt: treatment; PE: plant-bioactives extract; Trt × PE: interaction between treatment and plant-bioactives extract. SEM: standard error of the mean.

3.2. Effect of Plant-Bioactives Extract Supplementation on In Vitro Nutrient Digestibility and Ammonia-Nitrogen Concentration

The PE supplementation did not affect the IVDMD in the different experimental diets except in the 80:20 and 60:40 diets, where adding PE to these styles reduced the IVDMD ($p < 0.01$, Table 4). However, IVOMD, IVNDFD, and IVADFD did not show any differences when PE was added, as compared with their corresponding groups without PE supplementation ($p > 0.05$, Table 4). The PE inclusion increased the NH₃-N concentration ($p < 0.01$) in 100:0 and 60:40, and it tended to increase in 40:60 ($p = 0.088$), but there was a non-significant numerical increase in 80:20 ($p = 0.106$) and 20:80 ($p = 0.32$) (Table 4).

Table 4. Effect of plant-bioactives extract (PE) supplementation on digestibility and ammonia-nitrogen in different feeding styles after 24 h In Vitro incubation ($n = 18$).

Parameter	Treatments ¹										SEM	p-Value		
	100:0		80:20		60:40		40:60		20:80			Trt	PE	Trt × PE
	0	20	0	20	0	20	0	20	0	20				
IVDMD ²	0.38	0.38	0.45	0.42 **	0.49	0.46 **	0.52	0.51	0.55	0.53	0.56	<0.001	0.001	0.10
IVOMD ³	0.43	0.41	0.51	0.49	0.57	0.52	0.59	0.59	0.64	0.61	0.02	<0.001	0.03	0.82
IVNDFD ⁴	0.36	0.36	0.40	0.38	0.38	0.32	0.36	0.34	0.32	0.35	0.01	0.61	0.56	0.76
IVADFD ⁵	0.21	0.24	0.25	0.21	0.21	0.23	0.20	0.23	0.22	0.23	0.01	0.97	0.62	0.64
NH ₃ -N ⁶ (mg/dL)	4.46	6.43 ***	6.30	7.09	6.14	7.51 **	6.01	6.84	7.35	7.83	0.18	<0.001	<0.001	0.17

¹ grass:concentrate ratio; 0: 0 g PE/kg; 20: 200 g PE/kg of substrate. ² IVDMD: In Vitro dry matter digestibility. ³ IVOMD: In Vitro organic matter digestibility. ⁴ IVNDFD: In Vitro neutral detergent fiber digestibility. ⁵ IVADFD: In Vitro acid detergent fiber digestibility. ⁶ NH₃-N: ammonia-nitrogen. Asterisks in 20 mean significant difference between 0 and 200 g PE/kg in the same feeding model, ** ($p < 0.01$), *** ($p < 0.001$). Trt: treatment; PE: plant-bioactives extract; Trt × PE: interaction between treatment and plant-bioactives extract. SEM: standard error of the mean.

3.3. Effect of Plant-Bioactives Extract Supplementation on In Vitro Volatile Fatty Acids

The PE supplementation did not show any effect on the acetate concentration in all feeding models; however, the interaction between PE and treatment showed a difference for the PE supplemented groups to be increased in 100:0 ($p < 0.001$) and to be decreased in 20:80 ($p < 0.05$) compared with its corresponding treatment without PE inclusion (Table 5). In contrast, the acetate ratio decreased in all feeding models due to PE supplementation ($p < 0.01$), whereas the interaction between PE and treatment did not have a significant

effect ($p > 0.05$, Table 5). The concentration and the ratio of propionate and butyrate increased ($p < 0.001$) by adding PE in all feeding models. Additionally, the concentration of total volatile fatty acids (TVFA) showed the same finding (Table 5). The acetate/propionate (A/P) ratio decreased ($p < 0.001$) with PE supplementation in all feeding styles (Table 5).

Table 5. Effect of plant-bioactives extract (PE) supplementation on volatile fatty acids in different feeding styles after 24 h In Vitro incubation ($n = 18$).

Parameter	Treatments ¹										SEM	<i>p</i> -Value		
	100:0		80:20		60:40		40:60		20:80			Trt	PE	Trt × PE
	0	20	0	20	0	20	0	20	0	20				
Acetate (mmol/L)	62.54	66.02 **	64.81	66.12	64.81	65.66	65.61	65.91	66.80	64.15 *	0.54	0.45	0.21	0.01
Propionate (mmol/L)	15.24	20.19 ***	18.57	22.95 ***	19.38	24.34 ***	20.61	26.82 ***	22.21	26.78 ***	0.29	<0.001	<0.001	0.20
Butyrate (mmol/L)	7.14	10.86 ***	8.53	11.38 ***	9.01	12.36 ***	9.17	12.71 ***	9.84	12.89 ***	0.16	<0.001	<0.001	0.21
TVFA ² (mmol/L)	84.91	97.07 ***	91.92	100.45 ***	93.20	102.36 ***	95.40	105.44 ***	98.85	103.81 *	0.78	<0.001	<0.001	0.07
Acetate (mol/100 mol)	73.64	67.93 ***	70.53	65.67 ***	69.47	64.00 ***	68.72	62.39 ***	67.54	61.59 ***	0.30	<0.001	<0.001	0.07
Propionate (mol/100 mol)	17.95	20.80 ***	20.20	22.92 ***	20.86	23.86 ***	21.66	25.49 ***	22.46	25.92 ***	0.20	<0.001	<0.001	0.04
Butyrate (mol/100 mol)	8.41	11.27 ***	9.27	11.41 ***	9.67	12.14 ***	9.62	12.12 ***	10.00	12.50 ***	0.13	<0.001	<0.001	0.50
A/P ratio ³	4.11	3.29 ***	3.49	2.88 ***	3.34	2.69 ***	3.19	2.46 ***	3.02	2.40 ***	0.04	<0.001	<0.001	0.03

¹ grass:concentrate ratio; 0: 0 g PE/kg; 20: 200 g PE/kg of substrate. ² TVFA: total volatile fatty acids. ³ A/P: acetate/propionate. Asterisks in 20 mean significant difference between 0 and 200 g PE/kg in the same feeding model, * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). Trt: treatment; PE: plant-bioactives extract; Trt × PE: interaction between treatment and plant-bioactives extract. SEM: standard error of the mean.

4. Discussion

The CH₄ emissions from ruminants are not only a serious environmental issue but also a significant source of energy loss to the animals. Different kinds of antimethanogenic compounds have already been studied to investigate their potential to reduce CH₄ production [20]; however, there are limitations to their use due to their negative impacts on rumen fermentation characteristics [8], and they exhibited inconsistent efficiency with different feeding styles [9,21–23]. Therefore, sustainable and immediate CH₄ mitigation strategies for the livestock industry are in high demand. Combining different plant extracts to achieve effective and sustainable CH₄ reduction is a relatively new and promising approach [23]. The PE, a novel plant-based combination of garlic and citrus extracts, showed promising results when used as a feed supplement for CH₄ mitigation from ruminants [13,24]. Therefore, this study was performed to evaluate the efficacy of PE with different kinds of feeding styles in ruminants. Although the current study provides interesting information about the effect of PE on rumen fermentation, digestibility, and CH₄ production, the microbial analysis, which is very important for proper interpretation of the current findings, has not been done. Microbial characteristics are very important for obtaining the whole picture of fermentation, which was underlined by Pers-Kamczyc et al. [25]; however, our study, which we consider to be a pilot study, was carried out following the positive result of the tested PE effect on In Vitro rumen fermentation. Forthcoming research considering microbial analysis is strongly needed for a better understanding of the potential of this new mixture. Importantly, the finding that some of the differences in the results might arise from the differences in the nutrient composition due to PE addition rather than to the plant-bioactives present in PE must be taken into account when interpreting the current study.

Similar to the findings of the current study, PE increased gas production when used as a feed supplement with rumen fluid collected from sheep, which may reflect a stimulating effect of PE on rumen microbes [14]. This finding has been reported previously from a 48-h In Vitro gas production study conducted by Hansen and Nielsen [26]. Furthermore, PE increased the concentration of ruminal $\text{NH}_3\text{-N}$, which might be due to the role of PE in enhancing the proteolysis process. This nitrogen source can be captured and used by rumen microorganisms to build their own protein [27], which in turn would be used as a protein source for the animal [28]. A similar effect has been reported when this PE was used as a feed supplement with a 70:30 ratio of forage to concentrate diet in the rumen simulation technique (RUSITEC) [29]. The same finding has also been observed with garlic oil with a 50:50 ratio of forage to concentrate diet for 24 h incubation by Busquet et al. [30].

The PE supplementation did not interfere with fiber degradability in all feeding models, which was similar to the findings of García-González et al. [31], who reported that inclusion of garlic bulbs in the substrate in an In Vitro trial did not affect IVNDFD, and Zhong et al. [32], who declared that adding garlic powder to the basal diet did not change the NDF and ADF digestibility through an in vivo trial using lambs. Rumen microbiome analysis in upcoming studies would provide a better understanding of PE's effect on nutrient digestibility and proteolytic bacteria.

The synergism between the organosulfur compounds and flavonoids in the PE mixture was effective in decreasing CH_4 production in all feeding models. The reduction in CH_4 may be due to the direct inhibitory effect of PE on methanogenic archaea. In PE supplemented treatment, Eger et al. [13] and Ahmed et al. [14] reported a lower abundance of the family *Methanobacteriaceae*, which is the major CH_4 producer in the rumen. This was attributed to the toxicity of organosulfur compounds of garlic, such as diallyl sulfide and allicin, in inhibiting certain sulfhydryl-containing enzymes essential for the metabolic activities of methanogenic archaea [33]. Moreover, it has been reported that flavonoids have the ability to reduce CH_4 production as they have antimicrobial activities through interfering with cellular integrity of some bacteria as well as protozoa [34]. It has been established that ruminal ciliated protozoa could enhance methanogenesis, as they are major H_2 producers in the rumen and are in symbiotic relationships with methanogens [35]. Although the impact of PE on protozoa has not yet been investigated, allicin and flavonoids have shown toxic effects on protozoa [36,37]. Any effect of PE on protozoa has to be confirmed in additional studies.

It is well established that CH_4 formation has been positively associated with more acetate production and negatively associated with increased propionate production [38]. The allicin and flavonoids in PE were able to shift rumen fermentation toward less acetate and more propionate and butyrate. This increase in propionate may be due to the role of PE in increasing the abundance of the *Prevotellaceae* and *Veillonellaceae* families, which was confirmed by Ahmed et al. [14]. *Prevotellaceae* is one of the dominant families in rumen fluid, and it is well known to produce propionate by utilizing hydrogen (H_2) produced during the fermentation of carbohydrates [39]. This pathway is the main pathway for H_2 consumption, and it represents a competitive and alternative pathway to methanogenesis [40,41]. Moreover, the family *Veillonellaceae* showed high relative abundance due to the effect of flavonoids extracted from citrus [36], and it was associated with propionate production [42]. Supplementation of steers with garlic powder reduced the A/P ratio [43]. Similarly, the current study showed the same finding. An increase in butyrate was also associated with a reduction in CH_4 production when the basal diet of ewes was supplemented with garlic extract [44].

Reports about the effects of garlic and flavonoid components on TVFA are inconsistent. Some studies reported that they had no effect on TVFA [31,45–47], but others reported an adverse effect [30,36,48] using an In Vitro batch culture system. In contrast, in the current study, the PE formulation increased the production of TVFA, which suggests improved feed efficiency. This phenomenon has also been observed previously in studies using In Vitro batch culture [14] and the RUSITEC system [13]. That improvement in

TVFA production might have occurred because the produced H₂ from fermentation was utilized by microorganisms to produce more propionate and butyrate in PE supplemented groups, while in control groups, part of H₂ was utilized by methanogens to produce CH₄ (energy loss). Therefore, the PE supplemented groups were more effective in redirecting H₂ toward production of beneficial byproducts (energy source). Another theory is that PE stimulates the metabolic activity of some rumen microbes to utilize PE particles as feed, thus producing more TVFA—a theory that could be proven by demonstrating an increase in the production of total gas and CO₂. The latter theory could also be supported by a recent report that flavonoids could be used as a source of carbon for metabolism in the rumen [34]. This finding has to be confirmed and discovered in upcoming research.

5. Conclusions

In the present In Vitro study, we investigated the efficiency of the PE mixture on CH₄ production, rumen fermentation, feed efficiency, and digestibility in different feeding styles. According to the design of this In Vitro study, PE at a level of 20%/substrate had the potential to effectively reduce CH₄ production with all feeding styles. The PE showed a high reducing power up to 69% when the amount of concentrate composed up to 800 g/kg of the ration. Moreover, 20% PE supplementation improved the production of TVFA and shifted the fermentation profile toward less acetate and more propionate and butyrate. Additionally, PE did not impair fiber digestibility. Therefore, PE could be used as a feed supplement with all feeding styles to efficiently reduce CH₄ production by ruminants. The hypothesis and design of this trial should be taken into consideration when interpreting the experimental results. The PE dosage used in the current In Vitro trial would not be feasible for practical feeding. Further long-term in vivo trials with optimum dosage have to be done to confirm the current findings.

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Data Availability Statement: The data supporting reported results in this study are available on request from the corresponding author.

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