fermentation

MDPI

Article

In Vitro Fermentation and Degradation Characteristics of Rosemary Extract in Total Mixed Ration of Lactating Dairy Cows

Fanlin Kong, Shuo Wang, Zhijun Cao, Yajing Wang, Shengli Li and Wei Wang *

Beijing Engineering Technology Research Center of Raw Milk Quality and Safety Control, The State Key Laboratory of Animal Nutrition, Department of Animal Nutrition and Feed Science, College of Animal Science and Technology, China Agricultural University, No. 2 Yuanmingyuan West Road, Haidian District, Beijing 100094, China

* Correspondence: wei.wang@cau.edu.cn; Tel.: +86-010-62733789

Abstract: Rosemary extract (RE) is characterized as an antioxidant, and it has the potential to reduce methane emission and change microbial fermentation. Hence, to the extent of the evaluation of RE in ruminant nutrition, the in vitro fermentation technique was used to investigate the effects of RE on the fermentation characteristics of a total mixed ration (TMR) fed to dairy cows. Different doses of RE were added to the TMR to obtain different concentrations of antioxidants, including 0 (CON), 0.05 (LRE), and 0.10 g/kg (HRE). A total of 500 mg ground TMR was incubated in buffer solution and rumen fluid for 48 h at 39 °C. Nutrient degradability, gas production parameters, gas composition, fermentation parameters, and microbial composition were analyzed. The results showed that nutrient degradability and total volatile fatty acid concentration were not affected by the treatments. Furthermore, total methane production and proportion were depressed in a dose-dependent way. The RE increased the propionate concentration and proportion linearly and decreased the acetate concentration and proportion linearly. Finally, microbial diversity analysis showed that the richness and evenness indexes were unchanged by different treatments, while *Prevotella_1* was decreased and *Prevotella_7* was increased with RE supplementation. In conclusion, RE is an effective inhibitor of methane emission of microbial fermentation and changed the profile of volatile fatty acids with no disadvantageous effects on diet utilization.

Keywords: rosemary extract; gas production; microbiota; methane emission; degradability

Citation: Kong, F.; Wang, S.; Cao, Z.; Wang, Y.; Li, S.; Wang, W. In Vitro Fermentation and Degradation Characteristics of Rosemary Extract in Total Mixed Ration of Lactating Dairy Cows. *Fermentation* **2022**, *8*, 461. https://doi.org/10.3390/fermentation8090461

Academic Editor: Peixin Fan

Received: 16 August 2022
Accepted: 14 September 2022
Published: 15 September 2022

1. Introduction

Ruminants produce products via the complex rumen microbiota, which enables ruminants to digest structural carbohydrates, while the rumen microbiota also contributes to 18% of total anthropogenic emissions [1]. Hence, increasing consensus on emission reduction and emission reduction measures becomes more and more important. In recent years, plant extracts have shown their emission reduction potential as feed additives in the dairy industry [2,3].

Rosemary (*Rosmarinus officinalis* L.) originating from the Mediterranean region belongs to the *Lamiaceae* family. It contains diverse metabolites, and their identification by high-performance liquid chromatography and gas chromatography has revealed an abundant quantity of polyphenols such as carnosol, carnosic acid, and rosmarinic acid. Carnosic acid is the most abundant and has among the highest antioxidant activities [4,5]. Diverse rosemary extracts (REs) have been used widely in the medicine, food, and cosmetics industries, mainly for their antioxidant, antimicrobial, and anti-inflammatory activities [6,7]. In addition, many in vitro and in vivo studies have been conducted to investigate the effects of RE on methane emission, and the results have been inconsistent [8–10]. The potential mechanism may be due to modifying ruminal fermentation by enhancing the efficiency

of energy utilization while decreasing methane emissions [11]. Despite this, very little is known about the effects of RE on methane emission in dairy cows.

Except for methane emission, the microbiota in the rumen mainly takes the responsibility for nutrient degradability, and the steady state of the rumen environment is important for production and health. However, the antibacterial effect of RE has been widely proven in food preservation. Carnosic acid has the most effectiveness against bacteria compared to other substrates in RE [12]. The synergy between different compositions and dose-dependent effects also exists in the antimicrobial activity of these active constituents [13,14]. Hence, RE as an anti-methanogenic plant product should be based on no adverse effects on rumen fermentation or nutrient degradability. The in vitro fermentation technique (IVFT) has been used widely to assess the nutritive value of different rations, feedstuffs, feed additives, and so on [15]. Although many factors influence the results in the IVFT experiments and the IVFT does not guarantee that the significant results in the IVFT experiment reappear in the in vivo experiments, the IVFT provides a more timesaving and inexpensive way to determine the effects of various treatments [15].

Considering the potential role of RE in methane emission and interaction with microorganisms, we speculated that RE supplementation may change microbial fermentation in this experiment. Thus, the objective of this study was to evaluate the effects of RE on (1) gas production and composition; (2) nutrient degradability; and (3) bacterial diversity and composition. Based on the results of this in vitro study, we provide fundamental information on RE in dairy production.

2. Materials and Methods

2.1. Experimental Design

2.1.1. Preparation of Rosemary Extract

The RE product was purchased from Hunan zhizhiyuan Co., Ltd. (Changsha, China). The active components were quantified by UPLC-MS/MS. Chromatographic separation was performed on a Waters Acquity H-class UPLC system with the column oven temperature maintained at 40 °C, using an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 µm particle size) (Waters, Milford, MA, USA). The UPLC system was coupled to a Micromass Xevo TQ-S triple quadrupole mass spectrometer (Waters, Manchester, UK) fitted with an electrospray ionization source in negative mode (ESI-). Analytical standards of carnosic and rosmarinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The RE had the following functional composition: 42.28 g/kg carnosic and 1.59 g/kg rosmarinic acid. The UPLC-MS/MS chromatograms are presented in Supplementary Figure S1.

2.1.2. In Vitro Process

Three lactating Holstein dairy cows (36.3 kg/day milk yield, 171 ± 18 days in milk, 2 parity) with rumen fistula were used to collect rumen fluid. Briefly, about 3 L of rumen content from the individual donor was collected from the fistula before morning feeding. Then, we put the rumen content into a steel artificial squeezer, and the rumen fluid was juiced by pressure. After that, all rumen fluid was transferred to a prewarmed vacuum bottle and cultivated in the laboratory with CO_2 at 39 °C until use. We measured the pH of the rumen fluid after sampling, and the average pH was 6.1 ± 0.31 (mean ± SD). The ingredients and nutrient composition of the total mixed ration (TMR) fed to the donor are presented in Supplementary Table S1.

A total of 15 kg TMR was sampled from the farm and dried. The TMR was the same as the TMR fed to the donors. Then, 5.79 g RE was mixed with 5 kg TMR to obtain a substrate with a 0.05 g/kg total antioxidant concentration (LRE). A total of 11.58 g RE was mixed with 5 kg TMR to obtain a substrate with a 0.10 g/kg total antioxidant concentration (HRE). Finally, 5 kg TMR with no RE supplementation was the control group (CON).·

The glass bottles (120 mL) were used in the in vitro experiment combined with a 75 mL fermentation system including 50 mL buffer solution and 25 mL rumen fluid. The buffer solution was formulated as previous description [16]. The rumen fluid was a mixture of

individual rumen fluid from three donors. A total of 500 mg TMR with different total antioxidant concentrations were used as fermentation substrates. The substrate, buffer, and rumen fluid were added to the bottles in order. After that, the bottles were immediately sealed by a rubber stopper after introducing anaerobic N_2 for 5 s. Six runs were conducted, and each run had 18 bottles. Then, 18 bottles were divided into 3 groups indicating 6 bottles in each group, in which 3 bottles were connected to the rumen simulation system for gas production recording (AGRSIII, Beijing, China) and another 3 bottles were used for the gas collection by pre-emptied airbags. All bottles were cultivated at 39 °C in a temperature incubator.

2.2. Gas Production

For the gas composition, a 1.0 mL gas subsample of each bottle was removed from individual airbags, and the gas composition of CH_4, CO_2, and H_2 was measured by a gas chromatographic method (GC522, Wufeng Instruments, Shanghai, China) [17]. Briefly, the gas chromatograph was equipped with a thermal conductivity detector and a 2 m stainless steel column packed with TDX-1. N_2 was the carrier gas, and the temperatures of the injector oven, column oven, and detector were 150, 80, and 200 °C.

2.3. In Vitro Degradability

After 48 h of fermentation, the solid proportion from 6 bottles per run per group was collected into individual nylon bags. After drying at 65 °C for 48 h, the solid proportion was used to analyze apparent nutrient degradability. The dry matter, neutral detergent fiber, acid detergent fiber, and crude protein contents were determined according to Hao et al. [18].

2.4. Fermentation Parameters

Fermentation fluid in each bottle was also saved into 2.5 mL microtubes for microbiota analysis, and another 15 mL was used to analyze volatile fatty acid (VFA) and NH_3-N concentrations. The pH value was measured immediately by a portable pH meter (S2-Meter, Mettler Toledo International Co., Ltd., Shanghai, China). The fermentation fluid for VFA analysis was measured by gas chromatography (Agilent 6890N, Agilent Technologies, Inc., Beijing, China), and NH_3-N was measured by a Multiskan SkyHigh microplate reader (Thermo Fisher Scientific, Shanghai, China) according to Kong et al. [16]. For VFA analysis, a 15 m semicapillary column packed with chromosorb 101 was equipped with gas chromatography with N2 as the carrier gas. The crotonic acid was the internal standard at a column temperature of 120 °C. Iso-butyrate and iso-valerate were summed as branched-chain volatile fatty acid (BVFA).

2.5. Microbial Analysis

For microbial diversity analysis, the sequencing process was conducted according to the previous description [19]. Briefly, bacterial DNA was extracted using an Omega Stool DNA kit (Omega Bio-Tek, Norcross, GA, USA). The universal primers 338F and reverse primers 306R were used to perform amplicon library preparation. After amplification, the amplicons were electrophoresed on 2% agarose gel and purified using an Agencourt AM Pure XP kit (Beckman Coulter Genomics, Indianapolis, IN, USA) and quantified using the Quanti-FluorTM-ST system (Promega, Madison, MI, USA). Finally, the amplicons were pooled and sequenced on an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA). The raw data were filtered and processed using the Quantitative Insights Into Microbial Ecology 2 [20]. Amplicon sequence variants were generated using the DADA2 workflow following the removal of primers and chimeric and low-quality sequences. The taxonomic classification was performed using the SILVA database based on 99% sequence similarity [21]. All sequences were deposited in the NCBI Sequence Read Archive under the project number (PRJNA699978, SRR21068357-SRR21068374).

2.6. Statistical Analysis

The gas production data were downloaded from AGRSIII in Excel and then processed by the nonlinear regression procedure in the SAS software (version 9.4) according to the exponential model as described by France et al. [22].

$$GP = A \times \left[1 - e^{-C \times (time - Lag)}\right] \quad (1)$$

where GP is the gas production, A is the ideal maximum gas production, C is the gas production rate, and Lag is the lag phase before gas production commences. After the procedure, the time taken to reach half of the ideal maximum gas production (HT) and the average gas production rate when half of the ideal maximum gas production was produced (AGPR) were calculated according to the model below:

$$HT = \log\left(\frac{2}{C}\right) + Lag, \quad (2)$$

$$AGPR = \frac{A \times C}{2 \times (\log(2) + C \times Lag)}, \quad (3)$$

The mixed procedure from SAS 9.4 (SAS Institute, Inc., Cary, NC, USA) was used to analyze the data [23]. The model for the gas production parameter, nutrient degradability, gas composition, and fermentation parameters' data is presented as follows:

$$Y_{ijk} = \mu + Trt_i + Run_j + \varepsilon_{ijk} \quad (4)$$

where Y_{ijk} is the variable; μ is the population mean; Trt_i is the treatment effect; Run_j is the random run; ε_{ijk} is the random error. The Tukey–Kramer method was used for multiple comparisons. A SAS macro called "pdix800" was used to group treatment means with letters. The contrast statement was used for orthogonal polynomial contrasts. The significance threshold was set at 0.05, and the tendency was declared at $0.05 \leq p \leq 0.10$.

For microbial diversity analyses, principal coordinated analysis (PCoA) and an analysis of similarities (ANOSIM) using Bray–Curtis matrices were performed to test the statistical differences between different groups. The effects of RE supplementation on the α diversity indices of microbiota were assessed using the Kruskal–Wallis test. Differences were considered significant at $p < 0.05$. The linear discriminant analysis (LDA) effect size (LEfSe) tool was developed to detect features with significant differential abundance using the Kruskal–Wallis sum-rank test and the effect size value.

3. Results

3.1. Gas Production

The gas production parameters are shown in Table 1. No matter the different doses of RE supplementation, RE supplementation did not affect the gas production parameters ($p > 0.05$). The total GP was not affected ($p > 0.05$), and the AGPR trended to increase in a quadratic way ($p < 0.10$).

The gas composition and production obtained from HPGC analysis are presented in Table 2. RE supplementation increased the proportion and production of CO_2 and decreased the proportion and production of CH_4 linearly ($p < 0.05$). The CH_4 production expressed per unit substrate in the LRE and HRE groups was lower than that in the CON group ($p < 0.05$). For H_2, the proportion and production were not affected by treatment ($p > 0.05$).

Table 1. Effects of different doses of rosemary extract on gas production parameters of in vitro fermentation.

Items	Groups			SEM	*p*-Value		
	CON	LRE	HRE		G	L	Q
GP, mL/g	140.99	138.15	145.82	2.854	0.12	0.28	0.18
A, mL/g	142.37	139.75	160.34	6.499	0.07	0.10	0.20
C, h^{-1}	0.04	0.04	0.06	0.017	0.46	0.52	0.60
HT, h	1.76	1.94	1.81	0.075	0.14	0.61	0.14
AGPR, mL/h	49.58	41.16	58.16	4.732	0.25	0.25	0.07

CON, no supplementation; LRE, low-dose rosemary extract supplementation; HRE, high-dose rosemary extract supplementation, G, group effect; L, linear effect; Q, quadratic effect; GP, gas production; A, ideal maximum gas production; C, the gas production rate; HT, time to reach half the ideal maximum gas production; AGPR, average gas production rate when half of the ideal maximum gas is produced.

Table 2. Effects of different doses of rosemary extract on gas production of in vitro fermentation.

Items	Groups			SEM	*p*-Value		
	CON	LRE	HRE		G	L	Q
CO_2, %	77.40 ^{b}	79.77 ^{a}	80.29 ^{a}	0.244	<0.01	<0.01	0.02
CH_4, %	12.50 ^{a}	10.08 ^{b}	9.52 ^{b}	0.279	<0.01	<0.01	0.03
H_2, %	10.10	10.16	10.19	0.172	0.94	0.75	0.96
CO_2, mL/g	109.11	110.21	117.09	2.288	0.11	0.04	0.34
CH_4, mL/g	17.64 ^{a}	13.92 ^{b}	13.88 ^{b}	0.623	0.01	0.01	0.05
H_2, mL/g	14.23	14.03	14.85	0.195	0.06	0.07	0.08

CON, no supplementation; LRE, low-dose rosemary extract supplementation; HRE, high-dose rosemary extract supplementation, G, group effect; L, linear effect; Q, quadratic effect. Means within the same row with different superscripts are significantly different ($p < 0.05$). The same below.

3.2. In Vitro Degradability

Table 3 shows the effects of different doses of RE on nutrient degradability. DM, NDF, ADF, and CP degradability were not affected by RE supplementation ($p > 0.05$). Furthermore, there was no significant difference between the LRE and HRE groups ($p > 0.05$).

Table 3. Effects of different doses of rosemary extract on nutrient degradability measured in in vitro fermentation.

Items	Groups			SEM	*p*-Value		
	CON	LRE	HRE		G	L	Q
DM, %	52.97	53.03	53.45	2.723	0.91	0.90	0.96
NDF, %	45.33	44.47	43.45	1.762	0.51	0.50	0.99
ADF, %	41.30	38.56	41.14	2.292	0.22	0.59	0.24
CP, %	62.49	62.59	63.12	2.413	0.86	0.98	0.94

CON, no supplementation; LRE, low-dose rosemary extract supplementation; HRE, high-dose rosemary extract supplementation, G, group effect; L, linear effect; Q, quadratic effect; DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein.

3.3. Fermentation Parameters

The fermentation parameters including the pH value, NH_3-N, concentration, and proportions of VFA are shown in Table 4. There was no significant difference in the pH value, concentrations of NH_3-N, and total volatile fatty acid (TVFA) ($p > 0.05$). The RE supplementation trended to decrease the TVFA concentration ($p < 0.10$). Furthermore, RE supplementation decreased the concentrations and proportions of acetate and butyrate linearly and increased the concentration and proportion of propionate linearly ($p < 0.05$). Finally, the concentration and proportion of BVFA were reduced linearly ($p < 0.05$), and the valerate concentration was not changed by RE supplementation ($p > 0.05$).

Table 4. Effects of different doses of rosemary extract on in vitro fermentation parameters.

Items	Groups			SEM	*p*-Value		
	CON	LRE	HRE		G	L	Q
pH value	6.21	6.19	6.18	0.021	0.86	0.52	0.83
NH_3-N, mg/dL	16.81	16.53	17.79	0.423	0.11	0.12	0.15
Concentration, mmol/L							
TVFA	89.98	81.16	81.35	3.332	0.17	0.08	0.28
Acetate	57.76 ^{a}	48.80 ^{b}	44.79 ^{b}	2.476	<0.01	<0.01	0.42
Propionate	19.31 ^{b}	20.60 ^{b}	25.27 ^{a}	0.921	<0.01	<0.01	0.15
Butyrate	9.31 ^{a}	7.60 ^{b}	7.16 ^{b}	0.431	<0.01	<0.01	0.24
Valerate	1.51	1.52	1.57	0.032	0.24	0.83	0.09
BVFA	2.08 ^{b}	2.59 ^{a}	2.61 ^{a}	0.109	<0.01	<0.01	0.05
Molar proportion, %							
Acetate	64.14 ^{a}	59.85 ^{b}	54.99 ^{c}	0.954	<0.01	<0.01	0.819
Propionate	21.53 ^{c}	25.40 ^{b}	31.13 ^{a}	0.741	<0.01	<0.01	0.314
Butyrate	10.35	9.48	8.80	0.467	0.06	0.03	0.861
Valerate	1.69 ^{b}	2.00 ^{a}	1.87 ^{ab}	0.085	0.04	0.14	0.05
BVFA	2.30 ^{b}	3.28 ^{a}	3.21 ^{a}	0.137	<0.01	<0.01	<0.01

CON, no supplementation; LRE, low-dose rosemary extract supplementation; HRE, high-dose rosemary extract supplementation, G, group effect; L, linear effect; Q, quadratic effect; TVFA, total volatile fatty acid; BVFA, branched-chain volatile fatty acid.

3.4. Microbial Diversity

Figure 1 shows an overview of microbiota diversity. For the α diversity index, RE supplementation did not affect the richness of microbiota (ACE index) in Figure 1A ($p > 0.05$). The Shannon index also did not change, indicating that RE supplementation did not affect the evenness of microbiota ($p > 0.05$). However, there was a distinct separation between the RE supplementation groups and the CON group in the PCoA plot based on Bray–Curtis, and PREANOVA analysis also showed a significant difference ($p < 0.05$).

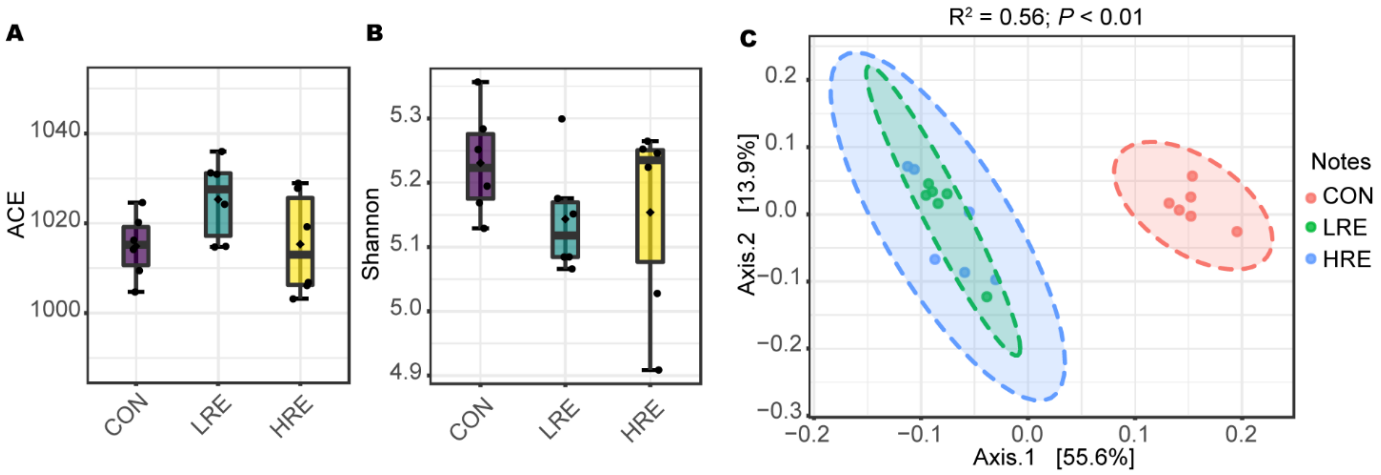

Figure 1. Effects of different doses of rosemary extract on the diversity of microbiota by in vitro fermentation technique. The α diversity indexes including the ACE (**A**) and Shannon indexes (**B**) were calculated based on the OTU level. Principal coordinate analysis (PCoA) combined with non-parametric multivariate analysis of variance (PREANOVA) was calculated based on the OTU level and Bray–Curtis distances (**C**). CON, no supplementation; LRE, low-dose rosemary extract supplementation; HRE, high-dose rosemary extract supplementation.

The figure below shows the main genera among the individual samples (Figure 2). Only the top 15 genera were included. *Prevotella_1*, *Selenomonas*, *Prevotella_7*, *Rikenellaceae_RC9_gut_group*, *Succiniclasticum*, *Succinivibrionaceae_UCG_001*, *Selenomonas_1*, *Anaerovibrio*, *Succinivibrio*, and *Megasphaera* showed more than 1% abundance in all samples.

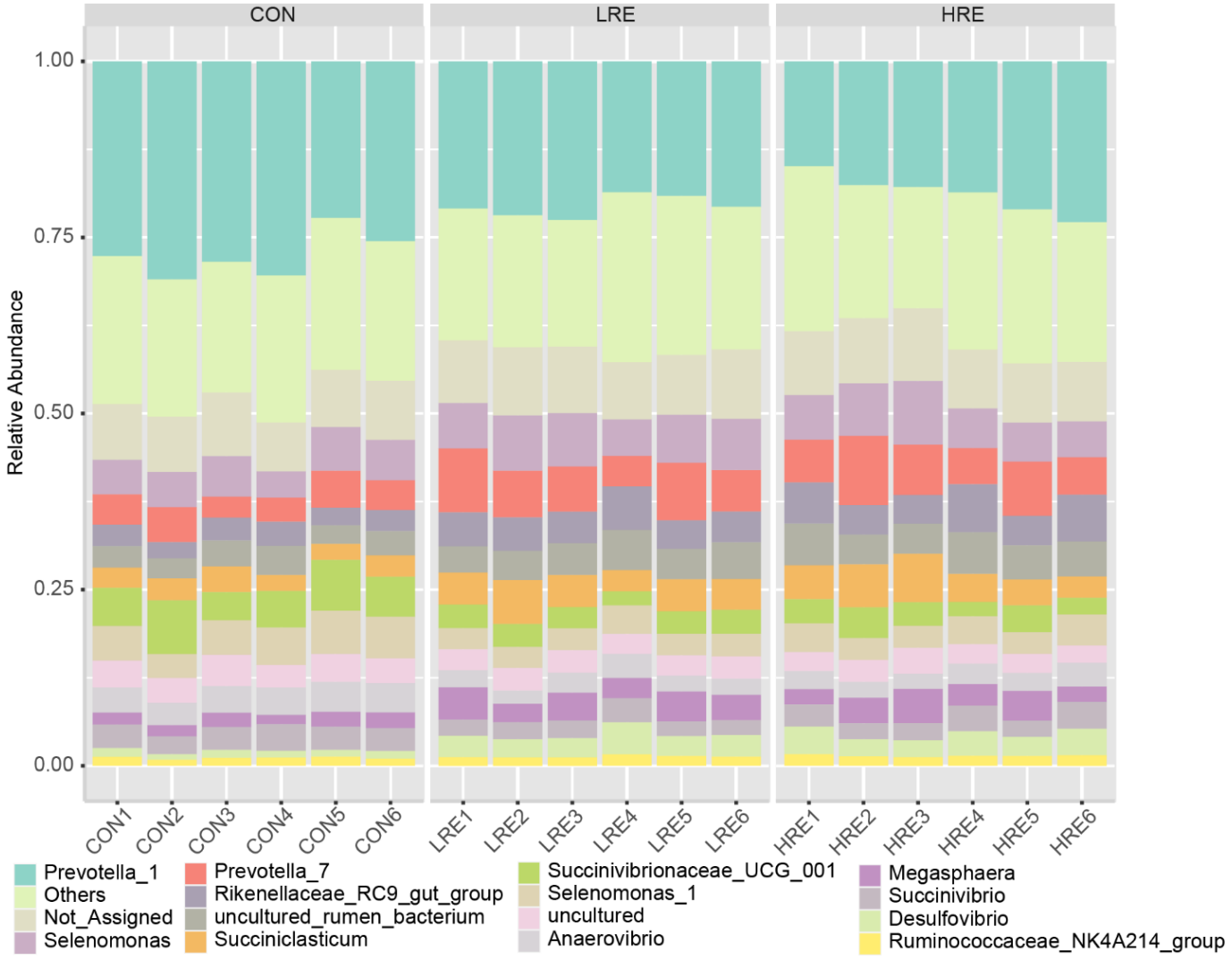

Figure 2. Genera composition of the microbiota from in vitro fermentation technique. Only the top 15 abundance genera were included in this figure. CON, no supplementation; LRE, low-dose rosemary extract supplementation; HRE, high-dose rosemary extract supplementation.

The LEfSe analysis was used to filter significant genera, and the results are show in Figure 3. A total of 16 genera were filtered by the LEfSe analysis, in which 8, 3, and 4 genera were the significant features from the CON, LRE, and HRE groups. The abundances of *Prevotella_1*, *Prevotella_7*, *Rikenellaceae_RC9_gut_group*, *Succiniclasticum*, *Succinivibrionaceae_UCG_001*, *Selenomonas_1*, *Anaerovibrio*, and *Megasphaera* were higher than 1% ($p < 0.05$. LDA score > 2).

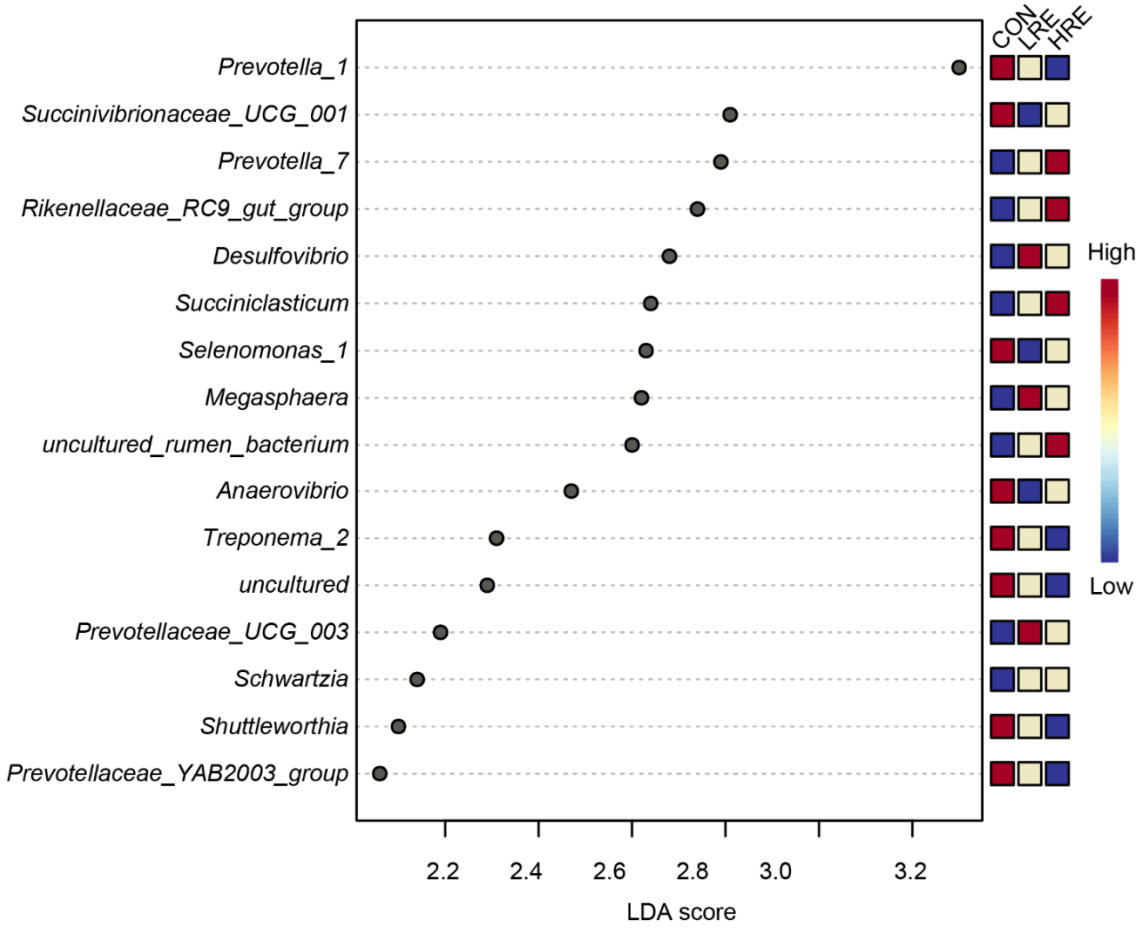

Figure 3. Linear discriminant analysis effect size (LEfSe) at the genus level. A cut-off value ≥ 2 of the LDA score was used to filter significant genera, and the adjusted *p*-value cut-off was <0.05. CON, no supplementation; LRE, low-dose rosemary extract supplementation; HRE, high-dose rosemary extract supplementation.

4. Discussion

In vitro fermentation techniques have been widely applied to evaluate the effects of feed additives on ration fermentation and methane production [15]. In the current study, unchanged gas production and nutrient degradability in response to RE were observed. The effect of RE on rumen fermentation has been reported, and the results have been inconsistent. For example, Cobellis et al. [24] observed that supplementation of rosemary essential oil (EO) decreased dry matter and neutral detergent fiber degradability, and gas production decreased with increasing rosemary EO supplementation. Tawfeeq et al. [25] indicated that rosemary leaves and watery and alcoholic extractions had no effects on digestibility, while rosemary organic extraction significantly decreased dry matter digestibility and organic digestibility. Multiple factors (e.g., diet, donor, and time) have interaction effects on in vitro experiments, and these factors should be considered. However, none of these studies considered the effect of RE on the TMR of lactating dairy cows.

Although the total gas production was not changed, the gas profile was changed by RE supplementation. Methane production is a fundamental rumen process; it could account for 2–12% of energy consumption in feed [26], and CO_2 and H_2 are the precursors to produce methane [27]. The changes in gas composition may be explained by the changes in VFA. The formation of acetate is associated with electron release. In contrast, propionate and butyrate are responsible for electron sinks in the rumen ecosystem. Glucose fermentation to two propionates results in the net use of electrons (equivalent to two H_2), and glucose fermentation to butyrate results in only two H_2 compared with the four H_2 from glucose

fermentation to acetate [27]. Hence, RE supplementation may reduce the total H_2 production by facilitating propionate production. However, the H_2 concentration and proportion were not changed by RE supplementation. We speculated that the utilization of H_2 was also impacted by RE supplementation and led to H_2 accumulation. In this experiment, the methanogens were not measured. Cobellis et al. [28] found that rosemary leaves decreased the abundance of archaea. A similar in vitro experiment that used rumen fluid from beef found that rosemary EO also decreased the acetate and butyrate proportions and increased the propionate and valerate proportions [29]. Several in vivo experiments feeding different forms of rosemary to goats or sheep obtained the same variation and proved the in vitro results [30,31]. Hence, further animal experiments are needed to determine the RE effects on milk performance and rumen fermentation. Interestingly, the BVFA concentration and proportion increased after RE supplementation. Branched-chain amino acids including leucine, isoleucine, and valine are the precursors of BVFA [32]. The UPLE-MS analysis revealed that rosemary infusions contained isoleucine, which is the most abundant amino acid [33].

The function of these plant extracts depends on their composition, and their composition is highly variable. A meta-analysis concluded on the effects of EO on ruminal fermentation of sheep and found that carnosic acid is the major bioactive compound [34], which was consistent with the UPLC-MS/MS results of RE. Furthermore, the replacement of rosemary leaves in sheep reduced the abundance of archaea, which may contribute to the reduction of methane emission [28]. However, the abundance of bacteria and protozoa was not affected [28]. In our study, metagenome analysis was not conducted to detect different microorganisms, while several indies including the unchanged pH value, ACE index, and Shannon index demonstrated that rumen fermentation still kept a steady state, and the effects of RE on gas production, TVFA concentration, and nutrient degradability also support this suggestion regardless of the doses.

Although the deposition of carnosic acid and carnosol in lamb tissues confirmed the escape of RE from ruminal fermentation [35], the microbial diversity analysis in our study supports the direct influences of RE on rumen microbiota. For the 16 bacterial genera whose relative abundance was affected by RE supplementation, half of these genera showed a $\geq$ 1% relative abundance. The abundance of *Prevotella* was significantly decreased in the presence of rosemary leaves [28], which is consistent with our results because *Prevotella_1* and *Prevotella* are generally considered major producers of propionate and consume a large range of nutrients [36]. Whole-rumen contents' exchange between high and low production efficiency to assess the response of ruminal bacterial and fungal microbiota showed that *Prevotella_1* was associated with high-efficiency cows and *Prevotella_7* was enriched in low-efficiency cows [37]. Furthermore, when evaluating the inhibiting effects of ozone on methane production, the other *Prevotella spp.*, *Prevotella_YAB2003*, had a positive correlation with propionate concentration [38]. *Succinivibrionaceae_UCG-001* is also a core microbiome in dairy cows and is characterized by succinate [39]. These changes of *Prevotella* support a decreasing propionate concentration, which is inconsistent with our results. The higher propionate concentration and molar proportion may be explained by the higher relative abundance of *Rikenellaceae_RC9_gut_group*, which has a role in structural carbohydrate degradation and produces succinate, a propionate precursor [40]. Several genera, *Succiniclasticum* and *Schwartzia,* belonging to the order *Selenomonadales*, were also reported to be correlated with propionate production in the rumen of dairy cows [41,42]. Whether those genera were affected by RE directly or changed by other microorganisms remains unclear. Our results showed that RE increased propionate production.

The study showed that the abundance of *Anaerovibrio* was enriched in the CON group and decreased with RE supplementation. *Anaerovibrio* is a well-known rumen lipolytic bacterium [43,44]. Hence, RE supplementation may have the potential to reduce lipid degradability. Our result showed that acetate decreased with RE supplementation. *Treponema spp.* are commonly distributed in the gastrointestinal tract of ruminants, encode a wide variety of carbohydrate-active enzymes, and act synergistically with cellulolytic

bacteria to degrade cellulose and degrade plant fibers, such as xylans [45]. Although the NDF and ADF degradability was not significantly changed in the present study, indeed, NDF degradability decreased with the addition of RE numerically.

5. Conclusions

In this experiment, the RE supplementation reduced methane emission in a dose-dependent way with no changes in nutrient degradability. The profile of VFA was also consistent with the result of gas composition, in which the RE supplementation increased the propionate concentration and decreased the acetate concentration. Although the composition of the microbiota was changed, RE supplementation did not affect the richness and evenness of the microbiota. The changed profile of VFA would provide a different substrate for milk synthesis. These results could be used for the application of RE in the ration of ruminants. Hence, our study provided preliminary results on RE. However, additional in vivo studies should be conducted to test these effects.

Supplementary Materials: The following Supporting Information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8090461/s1, Table S1: The ingredients and nutrient composition of the total mixed ration; Figure S1: UPLC-MS/MS chromatograms of rosemary extract.

Author Contributions: Conceptualization, W.W. and S.L.; methodology, Z.C.; resources, Y.W. and S.W.; writing—original draft preparation, F.K.; writing—review and editing, F.K.; supervision, W.W.; funding acquisition, S.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the China Agricultural Research System of Ministry of Finance and Ministry of Agriculture and Rural Affairs (CARS36).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: The study was conducted according to the guidelines of the Declaration of the Beijing Association for Science and Technology (ID No. SYXK, 2016-0008) and approved by the Institutional Ethics Committees of China Agricultural University (XXMB-2020-12-03-2).

Data Availability Statement: All sequences have been deposited in the NCBI Sequence Read Archive under the project number (PRJNA699978, SRR21068357- SRR21068374).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mizrahi, I.; Wallace, R.J.; Moraïs, S. The rumen microbiome: Balancing food security and environmental impacts. *Nat. Rev. Microb.* **2021**, *19*, 553–566. [CrossRef]
2. Kim, E.T.; Kim, C.H.; Min, K.S.; Lee, S.S. Effects of Plant Extracts on Microbial Population, Methane Emission and Ruminal Fermentation Characteristics in In vitro. *Asian-Australas. J. Anim. Sci.* **2012**, *25*, 806–811. [CrossRef]
3. Kolling, G.J.; Stivanin, S.C.B.; Gabbi, A.M.; Machado, F.S.; Ferreira, A.L.; Campos, M.M.; Tomich, T.R.; Cunha, C.S.; Dill, S.W.; Pereira, L.G.R.; et al. Performance and methane emissions in dairy cows fed oregano and green tea extracts as feed additives. *J. Dairy Sci.* **2018**, *101*, 4221–4234. [CrossRef] [PubMed]
4. Okamura, N.; Fujimoto, Y.; Kuwabara, S.; Yagi, A. High-performance liquid chromatographic determination of carnosic acid and carnosol in Rosmarinus officinalis and Salvia officinalis. *J. Chromatogr. A* **1994**, *679*, 381–386. [CrossRef]
5. Cuvelier, M.E.; Berset, C.; Richard, H. Antioxidant constituents in sage (*Salvia officinalis*). *J. Agr. Food Chem.* **1994**, *42*, 665–669. [CrossRef]
6. de Macedo, L.M.; Santos, É.M.d.; Militão, L.; Tundisi, L.L.; Ataide, J.A.; Souto, E.B.; Mazzola, P.G. Rosemary (*Rosmarinus officinalis* L., syn Salvia rosmarinus Spenn.) and Its Topical Applications: A Review. *Plants* **2020**, *9*, 651. [CrossRef]
7. Nieto, G.; Ros, G.; Castillo, J. Antioxidant and Antimicrobial Properties of Rosemary (*Rosmarinus officinalis*, L.): A Review. *Medicines* **2018**, *5*, 98. [CrossRef]
8. Delgadillo-Ruiz, L.; Bañuelos-Valenzuela, R.; Gallegos-Flores, P.; Echavarría-Cháirez, F.; Meza-López, C.; Gaytán-Saldaña, N. Modification of ruminal fermentation in vitro for methane mitigation by adding essential oils from plants and terpenoid compounds. *Abanico Vet.* **2021**, *11*, 1–12. [CrossRef]
9. Güney, M.; Karaca, S.; Erdogan, S.; Kor, A.; Kale, C.; Onalan, S.; Demirel, M.; Bingol, N.T. Effects of dietary supplementation with rosemary oil on methanogenic bacteria density, blood and rumen parameters and meat quality of fattening lambs. *Ital. J. Anim. Sci.* **2021**, *20*, 794–805. [CrossRef]

10. Molho-Ortiz, A.A.; Romero-Pérez, A.; Ramírez-Bribiesca, E.; Marquez-Mota, C.; Ramírez-Orejel, J.C.; Corona, L. PSXIV-13 Effect of phytochemicals on in vitro rumen fermentation and methane production. *J. Anim. Sci.* **2019**, *97*, 445–446. [CrossRef]
11. Joch, M.; Cermak, L.; Hakl, J.; Hucko, B.; Duskova, D.; Marounek, M. In vitro screening of essential oil active compounds for manipulation of rumen fermentation and methane mitigation. *Asian Australas. J. Anim. Sci.* **2016**, *29*, 952. [CrossRef]
12. Vegara, S.; Funes, L.; Martí, N.; Saura, D.; Micol, V.; Valero, M. Bactericidal activities against pathogenic bacteria by selected constituents of plant extracts in carrot broth. *Food Chem.* **2011**, *128*, 872–877. [CrossRef]
13. Ivanovic, J.; Misic, D.; Zizovic, I.; Ristic, M. In vitro control of multiplication of some food-associated bacteria by thyme, rosemary and sage isolates. *Food Control* **2012**, *25*, 110–116. [CrossRef]
14. Moreno, S.; Scheyer, T.; Romano, C.S.; Vojnov, A.A. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radic. Res.* **2006**, *40*, 223–231. [CrossRef] [PubMed]
15. Yáñez-Ruiz, D.R.; Bannink, A.; Dijkstra, J.; Kebreab, E.; Morgavi, D.P.; O'Kiely, P.; Reynolds, C.K.; Schwarm, A.; Shingfield, K.J.; Yu, Z.; et al. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants—A review. *Anim. Feed Sci. Tech* **2016**, *216*, 1–18. [CrossRef]
16. Kong, F.; Lu, N.; Liu, Y.; Zhang, S.; Jiang, H.; Wang, H.; Wang, W.; Li, S. Aspergillus oryzae and Aspergillus niger Co-Cultivation Extract Affects In Vitro Degradation, Fermentation Characteristics, and Bacterial Composition in a Diet-Specific Manner. *Animals* **2021**, *11*, 1248. [CrossRef]
17. Zhang, D.F.; Yang, H.J. In vitro ruminal methanogenesis of a hay-rich substrate in response to different combination supplements of nitrocompounds; pyromellitic diimide and 2-bromoethanesulphonate. *Anim. Feed Sci. Tech.* **2011**, *163*, 20–32. [CrossRef]
18. Hao, Y.; Huang, S.; Liu, G.; Zhang, J.; Liu, G.; Cao, Z.; Wang, Y.; Wang, W.; Li, S. Effects of Different Parts on the Chemical Composition, Silage Fermentation Profile, In Vitro and In Situ Digestibility of Paper Mulberry. *Animals* **2021**, *11*, 413. [CrossRef] [PubMed]
19. Kong, F.; Liu, Y.; Wang, S.; Zhang, Y.; Wang, W.; Yang, H.; Lu, N.; Li, S. Nutrient Digestibility, Microbial Fermentation, and Response in Bacterial Composition to Methionine Dipeptide: An In Vitro Study. *Biology* **2022**, *11*, 93. [CrossRef] [PubMed]
20. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]
21. Pruesse, E.; Quast, C.; Knittel, K.; Fuchs, B.M.; Ludwig, W.; Peplies, J.; Glöckner, F.O. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* **2007**, *35*, 7188–7196. [CrossRef] [PubMed]
22. France, J.; Dijkstra, J.; Dhanoa, M.; Lopez, S.; Bannink, A. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: Derivation of models and other mathematical considerations. *Brit. J. Nutr.* **2000**, *83*, 143–150. [CrossRef]
23. Institute, S. *Base SAS 9.4 Procedures Guide: Statistical Procedures*; SAS Institute: Cary, NC, USA, 2017.
24. Cobellis, G.; Petrozzi, A.; Forte, C.; Acuti, G.; Orrù, M.; Marcotullio, M.; Aquino, A.; Nicolini, A.; Mazza, V.; Trabalza-Marinucci, M. Evaluation of the Effects of Mitigation on Methane and Ammonia Production by Using *Origanum vulgare* L. and *Rosmarinus officinalis* L. Essential Oils on in Vitro Rumen Fermentation Systems. *Sustainability* **2015**, *7*, 12856–12869. [CrossRef]
25. Tawfeeq, J.A.; Al-Omrani, H.A.; Shaker, R.M.; Hamza, Z.R.; Abbas, S.F.; Jabbar, R.H. Effect of Peppermint and Rosemary Extractions on Ruminant In-vitro Digestibility. *Adv. Anim. Vet. Sci.* **2019**, *7*, 910–913. [CrossRef]
26. Johnson, K.A.; Johnson, D.E. Methane emissions from cattle. *J. Anim. Sci.* **1995**, *73*, 2483–2492. [CrossRef] [PubMed]
27. Leahy, S.C.; Janssen, P.H.; Attwood, G.T.; Mackie, R.I.; McAllister, T.A.; Kelly, W.J. Electron flow: Key to mitigating ruminant methanogenesis. *Trends Microbiol.* **2022**, *30*, 209–212. [CrossRef]
28. Cobellis, G.; Yu, Z.; Forte, C.; Acuti, G.; Trabalza-Marinucci, M. Dietary supplementation of Rosmarinus officinalis L. leaves in sheep affects the abundance of rumen methanogens and other microbial populations. *J. Anim Sci. Biotechnol.* **2016**, *7*, 27. [CrossRef]
29. Castillejos, L.; Calsamiglia, S.; Martín-Tereso, J.; Ter Wijlen, H. In vitro evaluation of effects of ten essential oils at three doses on ruminal fermentation of high concentrate feedlot-type diets. *Anim Feed Sci. Tech.* **2008**, *145*, 259–270. [CrossRef]
30. Sahraei, M.; Pirmohammadi, R.; Payvastegan, S. The effect of rosemary (*Rosmarinus officinalis* L.) essential oil on digestibility, ruminal fermentation and blood metabolites of Ghezel sheep fed barley-based diets. *Span. J. Agric. Res.* **2014**, *12*, 448–454. [CrossRef]
31. Kholif, A.E.; Matloup, O.H.; Morsy, T.A.; Abdo, M.M.; Abu Elella, A.A.; Anele, U.Y.; Swanson, K.C. Rosemary and lemongrass herbs as phytogenic feed additives to improve efficient feed utilization, manipulate rumen fermentation and elevate milk production of Damascus goats. *Livest Sci.* **2017**, *204*, 39–46. [CrossRef]
32. Kaneda, T. Iso- and anteiso-fatty acids in bacteria: Biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* **1991**, *55*, 288–302. [CrossRef] [PubMed]
33. Peixoto, J.A.B.; Álvarez-Rivera, G.; Alves, R.C.; Costa, A.S.G.; Machado, S.; Cifuentes, A.; Ibáñez, E.; Oliveira, M.B.P.P. Comprehensive Phenolic and Free Amino Acid Analysis of Rosemary Infusions: Influence on the Antioxidant Potential. *Antioxidants* **2021**, *10*, 500. [CrossRef]
34. Torres, R.N.S.; Moura, D.C.; Ghedini, C.P.; Ezequiel, J.M.B.; Almeida, M.T.C. Meta-analysis of the effects of essential oils on ruminal fermentation and performance of sheep. *Small Rumin. Res.* **2020**, *189*, 106148. [CrossRef]

35. Jordán, M.J.; Castillo, J.; Bañón, S.; Martínez-Conesa, C.; Sotomayor, J.A. Relevance of the carnosic acid/carnosol ratio for the level of rosemary diterpene transfer and for improving lamb meat antioxidant status. *Food Chem.* **2014**, *151*, 212–218. [CrossRef] [PubMed]
36. Krause, D.O.; Denman, S.E.; Mackie, R.I.; Morrison, M.; Rae, A.L.; Attwood, G.T.; McSweeney, C.S. Opportunities to improve fiber degradation in the rumen: Microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* **2003**, *27*, 663–693. [CrossRef]
37. Cox, M.S.; Deblois, C.L.; Suen, G. Assessing the Response of Ruminal Bacterial and Fungal Microbiota to Whole-Rumen Contents Exchange in Dairy Cows. *Front. Microbiol.* **2021**, *12*, 665776. [CrossRef] [PubMed]
38. Zhao, L.; Caro, E.; Holman, D.B.; Gzyl, K.E.; Moate, P.J.; Chaves, A.V. Ozone Decreased Enteric Methane Production by 20% in an in vitro Rumen Fermentation System. *Front. Microbiol.* **2020**, *11*, 571537. [CrossRef]
39. De Oliveira Santos, E.; Thompson, F. *The Family Succinivibrionaceae*; Springer: Berlin/Heidelberg, Germany, 2014. [CrossRef]
40. Pitta, D.W.; Pinchak, W.E.; Dowd, S.E.; Osterstock, J.; Gontcharova, V.; Youn, E.; Dorton, K.; Yoon, I.; Min, B.R.; Fulford, J. Rumen bacterial diversity dynamics associated with changing from bermudagrass hay to grazed winter wheat diets. *Microb. Ecol.* **2010**, *59*, 511–522. [CrossRef]
41. Van Gylswyk, N. Succiniclasticum ruminis gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int. J. Syst. Evol. Mic.* **1995**, *45*, 297–300. [CrossRef]
42. Van Gylswyk, N.; Hippe, H.; Rainey, F. Schwartzia succinivorans gen. nov., sp. nov., another ruminal bacterium utilizing succinate as the sole energy source. *Int. J. Syst. Evol. Mic.* **1997**, *47*, 155–159. [CrossRef]
43. Liu, K.; Xu, Q.; Wang, L.; Wang, J.; Guo, W.; Zhou, M. The impact of diet on the composition and relative abundance of rumen microbes in goat. *Asian-Australas. J. Anim. Sci.* **2017**, *30*, 531–537. [CrossRef] [PubMed]
44. Huws, S.A.; Kim, E.J.; Lee, M.R.; Scott, M.B.; Tweed, J.K.; Pinloche, E.; Wallace, R.J.; Scollan, N.D. As yet uncultured bacteria phylogenetically classified as Prevotella, Lachnospiraceae incertae sedis and unclassified Bacteroidales, Clostridiales and Ruminococcaceae may play a predominant role in ruminal biohydrogenation. *Environ. Microbiol.* **2011**, *13*, 1500–1512. [CrossRef] [PubMed]
45. Liu, J.; Pu, Y.-Y.; Xie, Q.; Wang, J.-K.; Liu, J.-X. Pectin induces an in vitro rumen microbial population shift attributed to the pectinolytic Treponema group. *Curr. Microbiol.* **2015**, *70*, 67–74. [CrossRef] [PubMed]