THE USE OF PHYTOGENIC FEED ADDITIVES TO ENHANCE PRODUCTIVITY AND HEALTH IN RUMINANTS

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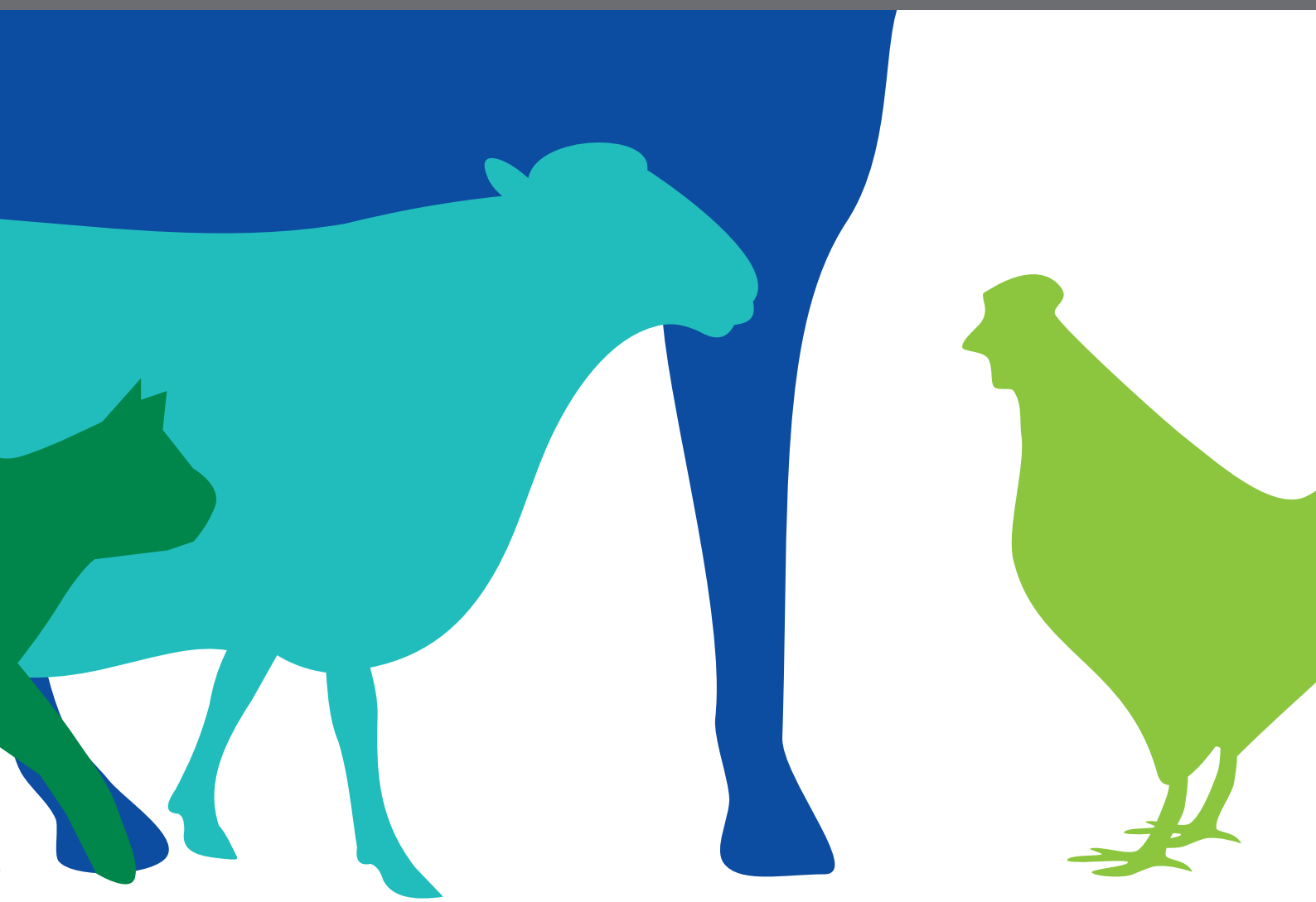

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THE USE OF PHYTOGENIC FEED ADDITIVES TO ENHANCE PRODUCTIVITY AND HEALTH IN RUMINANTS

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EDITORIAL
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Editorial: The Use of Phytogenic Feed Additives to Enhance Productivity and Health in Ruminants

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Editorial on the Research Topic

The Use of Phytogenic Feed Additives to Enhance Productivity and Health in Ruminants

Plant secondary metabolites (PSM) are biologically active compounds which can exert beneficial effects on ruminal fermentation, feed digestion, health, and productivity (1, 2). Due to their antimicrobial effects against undesirable ruminal bacteria, protozoa, and methanogens, PSM can serve as alternatives to antibiotic feed additives in ruminant production (1, 2). The special issue (12 articles: 9 research papers and 3 review articles) provides recent insights on new phytochemicals with potent activities, mechanisms of action, their synergistic effects, and interactions with other compounds.

Three reviews were published in this special issue. The review article by Hassan et al. discussed the ability of phytogenic additives to modulate rumen microbiome, fermentation kinetics and methanogenesis mediated through diet–microbe-phytochemical interactions. Authors concluded that pure PSM, plant extracts or PSM-rich phytogenic feeds have the ability to modulate rumen microbiota, increase volatile fatty acids and decrease ammonia and methane (CH_4) production. Inhibition of enteric CH_4 emission was consistently observed in *in vitro* experiments, while the *in vivo* effects varied greatly. The authors emphasized that many factors contribute to this vast variability, including variations of the chemical compositions and dose of the PSM, application methods, dietary composition, physiological stage of animals, feeding conditions, and progressive adaptation of microbes for specific phytochemicals. Moreover, they discussed the effects of production, extraction, processing, and application of phytochemicals on the expected responses. In another review, Sun summarized the effects of glucosinolates present in brassica forages on CH_4 emissions from ruminants, and concluded that brassica forages can be used as a useful tool to reduce CH_4 emitted per unit of DM intake compared with grass-based forages. The author explained physiological mechanisms in the mitigation of CH_4 emissions from ruminants fed brassica forages beyond the direct inhibitory effect on methanogens or fermentation profile changes. Glucosinolates and/or their ruminal microbial breakdown products are absorbed into the blood and then may stimulate the secretion of thyroid hormone causing many changes in rumen physiology including a reduction in ruminal digesta retention time, thereby reducing CH_4 emissions In the third review article, Tedeschi et al. reviewed the ecologically relevant phytochemicals including polyphenolics, terpenes antioxidants, alkaloids, flavonoids, condensed tannins and essential oils on ruminant performance, and their sustainable production and

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utilization to replace antibiotics. They recommended the use of many phytochemicals at the same time to obtain better biological responses in ruminant production.

The effect of phytogenic feed additive supplementation on *in vitro* fermentation was evaluated in 3 research articles. In one experiment, Ebeid et al. noted that *Camelina sativa* oil (0, 2, 4, 6, and 8% of dry matter) changed proportions of individual ruminal volatile fatty acids and decreased CH_4 production by altering total bacteria, protozoa, and methanogens populations. *Camelina sativa* oil caused a linear decrease in bacterial richness and evenness indices along with Shannon diversity. In another experiment, Ahmed et al. reported that Mootral [a commercial product containing *Allium sativum (*garlic) powder and *Citrus aurantium* (bitter orange) extracts] at 0, 10, and 20% of the substrate altered ruminal microbial community, produced more propionate, and reduce microbial groups associated with CH_4 production *in vitro*. Such effects were reflected as reducing CH_4 percentage and CH_4/CO_2 ratio in a dose-dependent manner. Moreover, Mootral increased abundances of H_2-consuming groups such as *Prevotellaceae* and *Veillonellaceae* and reduced some H_2-producing bacteria as well as reduced the major CH_4-producing family *Methanobacteriaceae* and increased *Methanomassiliicoccaceae*. Gomaa et al. reported that red osier dogwood extract at 1% of DM in the rumen simulated technique system tended to decrease acetate to propionate ratio and decreased starch disappearance in the barely-based diet, but not in the diet containing dried distillers grains with solubles. Rumen microbiota was not affected by red osier dogwood extract at the phylum level, but was altered at the genus level.

The response of ruminants to phytochemicals *in vivo* was evaluated in 6 studies. Hassan et al. observed that the supplementation of Murrah water buffaloes with a mixed phytogenics (ginger tuber, turmeric tuber, licorice roots, fennel seeds, fenugreek seeds, ajwain seeds, *Swertia chirata* leaves, *Terminalia chebula* fruit, *Citrullus colocynthis* fruit, and *Phyllanthus emblica* fruit in equal quantities) at 0, 15, 25, and 35 g/d for 6 weeks resulted in substantial changes in the rumen bacteriome composition (increased the abundances of *Firmicutes* and *Proteobacteria* phyla, and decreased the abundance of *Bacteroidetes* phylum and *Prevotella* genus) and milk fatty acid (increased *n*-3 fatty acid content and decreased stearic acid content). Heat stress in livestock causes reduction in production performance, immune responses, and health status as well as imbalances of stress-related hormones due to decreased antioxidant status, which can be ameliorated by supplementation of herbs (3). The study of Li et al. also showed that supplementation of mulberry leaf flavonoids (0, 15, 30, and 45 g/d) for 5 weeks, decreased the oxidative stress marker (e.g., malondialdehyde concentrations) and total antioxidant capacity and catalase while increasing the serum heat shock proteins, glutathione peroxidase, and insulin concentrations in Murrah buffaloes. The highest dose of mulberry leaf flavonoids at 45 g/d was the most appropriate dose for supplementation in lactating buffaloes to enhance lactation performance and alleviate heat-induced oxidative stress during the summer season. Additionally, Li et al. evaluated the effects of *Radix puerarin* extract supplementation for 60 days at 0, 200, 400, and 800 mg/kg in the feed concentrate and reported that *R. puerarin* extract at 400 mg/kg improved growth performance and meat quality of heat-stressed beef cattle by improving muscle total antioxidant status, superoxide dismutase, and glutathione peroxidase activity and reducing muscle fiber diameter, shear force and myosin heavy-chain (fast-glycolytic) muscle fiber composition. Galván et al. included a polyherbal feed mixture (*Achyrantes aspera, Trachyspermum ammi, Citrullus colocynthis, Andrographis paniculata*, and *Azadirachta indica*) in diets of growing calves at 0, 3, 4, and 5 g/d and noted that additives at 4 g/d showed the best performance results with improved growth and health status during the pre-ruminant to the weaning period through modification of different gene expression, notably biological processes associated with tight junction, mucin biosynthesis, and intestinal immunoglobulin A production. Moreover, they reported that polyherbal treatment could improve the metabolism of lipids, carbohydrates, proteins, and also immune response revealed through gene expression analysis. Petrič et al. evaluated the effect of a mixture of dry medicinal herbs (*Fumaria officinalis* L., *Malva sylvestris* L., *Artemisia absinthium* L., and *Matricaria chamomilla* L.) at 100 g DM/d and organic zinc at 70 mg Zn/kg diet on ruminal microbial fermentation and histopathology in lambs for 70 d. Supplements did not affect the ruminal fermentation parameters or the protozoal population of the lambs; however, they lowered total ruminal bacterial population. Additionally, the study showed that long-term dietary supplementation with organic zinc combined with a mixture of medicinal herbs with a strong antioxidant capacity could negatively affect the health of the ruminal epithelium. The findings highlight that the effect of dry medicinal herb mixtures depends on the variety and synergy of herbal polyphenols and the combination of bioactive compounds. Jiao et al. evaluated the effect of feeding cobalt and oregano essential oil blend in ram diets and reported improved cellulose and nutrient digestibility.

In summary, the research within this special issue, along with many other publications elsewhere, demonstrated the ability of phytogenic feed additives to modify nutrient digestion and ruminal microbes as well as improve health and host metabolism resulting in enhanced ruminant performance and abatement of CH_4 production. The effect of phytochemicals is dose-dependent revealing the importance of defining the optimal dose of each phytochemicals under specific conditions.

AUTHOR CONTRIBUTIONS

AK, UA, AP, and ZV have served as editors of the Research Topic and have co-written the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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A Mixed Phytogenic Modulates the Rumen Bacteria Composition and Milk Fatty Acid Profile of Water Buffaloes

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This study was aimed to evaluate the effect of a mixed phytogenic (MP) on rumen bacteria and their potential association with rumen fermentation and milk yield parameters in water buffaloes. Twenty Murrah buffaloes were fed a basal diet (consisting of maize silage, brewers' grains, and concentrate mixture) for 6 weeks supplemented with 0 (control), 15 (MP15), 25 (MP25), and 35 (MP35) g of mixed phytogenic/buffalo per d. The mixed phytogenic contained fennel (seeds), ajwain (seeds), ginger (tubers), *Swertia chirata* (leaves), *Citrullus colocynthis* (fruit), turmeric, fenugreek (seeds), *Terminalia chebula* (fruit), licorice (roots), and *Phyllanthus emblica* (fruit) in equal quantities. After 2 weeks of adaptation, daily milk yield, and weekly milk composition were recorded. On the last day of the experiment (d 42), rumen contents were collected to determine rumen fermentation parameters and bacterial diversity through 16S rRNA sequencing. Results revealed no change in dry matter intake, milk yield and rumen fermentation parameters except pH, which increased ($P = 0.029$) in response to MP supplementation. The mixed phytogenic increased ($P < 0.01$) milk fatty acids (C4 to C14:0) in MP15 only. The milk C16:1 content and its unsaturation index were higher ($P < 0.05$) in MP35 as compared to the control and other treatments. Furthermore, C18:3n3 was higher ($P < 0.05$) in the control, MP15, and MP25, as compared to MP35. Supplementation of MP tended to increase ($P = 0.095$) the Shannon index of bacterial alpha diversity and a difference ($P < 0.05$) among treatment groups was observed in beta diversity. Feeding MP increased the *Firmicutes, Proteobacteria,* and *Spirochaetes* but decreased *Bacteroidetes* numerically. In addition, the dominant genus *Prevotella* decreased in all treatment groups while *Pseudobutyrivibrio, Butyrivibrio,* and *Succinivibrioanceae* increased numerically in MP25 and MP35. The mixed phytogenic promoted groups of rumen bacteria positively associated with milk and fat yield. Overall, our study revealed 14 positive correlations of rumen bacteria with milk yield and eight with rumen fermentation parameters. Our findings reveal substantial changes in the rumen bacteriome composition and milk fatty acid content in response to MP but these results should be interpreted carefully, as the sample size of our study was relatively small.

Keywords: mixed phytogenic, rumen bacteria, rumen fermentation, milk yield, fatty acids, buffalo

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INTRODUCTION

Gut microbes perform major digestive and metabolic activities to derive energy from nutrient components of the diet and are considered one of the crucial factors affecting the feed conversion efficiency of ruminants. During rumen fermentation, fermentable dietary components are broken down into volatile fatty acids (VFA) and microbial protein (MCP) is synthesized. Volatile fatty acids and MCP satisfy a major part of the dietary energy (ca. 80%) and protein (65–85%) requirements of the host (1, 2). Since the availability of fermentation products (amount and composition) impacts milk yield, milk fat, and protein synthesis, rumen fermentation is considered to be a vital process, affecting the performance of dairy animals (3). The escape of microbial cells from the rumen is followed by their digestion and absorption in the small intestine leading to the availability of amino acids, needed to satisfy the requirements of the host animal (4). Cell membranes of rumen bacteria are composed of different fatty acids like odd and branch-chain fatty acids that also contribute to fatty acid profile of milk (5).

Some phytogenic feed additives, particularly secondary plant compounds, have shown to affect the composition of the rumen microbiome, change rumen fermentation dynamics and have impact on milk production performance (6–10). So far, the majority of *in vitro* and *in vivo* studies, aimed to evaluate the use of plant secondary metabolites in ruminants, have been conducted using one or two plants or their extract or essential oils. In contrast, we wanted to test if different plant-based compounds would act synergistically and therefore decided to supplement a relatively complex mixture of phytogenic compounds derived from 10 plants with proven antioxidant or antimicrobial activity. Combinations of phytogenic antioxidants have, for example, greater potential to scavenge free radicals than individual plant compounds (11).

The plant compounds selected for this study have previously shown to be bioactive and had beneficial effects on rumen fermentation and animal performance (12). For example, supplementation of ginger improved *in vitro* fermentation characteristics by reducing ammonia nitrogen (NH_3-N), methane and acetate to propionate ratio along with desirable effects on fibrolytic bacteria and protozoa (13). Turmeric (*Curcuma long*) possesses anti-bacterial (14), anti-parasitic (15) and antioxidant properties owing to its high content of curcumin and other curcuminoids (16). In a previous study, we highlighted the potential effects of curcumin as an epigenetic modulator with potential effects on animal physiology (17). Recently, curcumin supplementation has shown to increase milk yield and unsaturated fatty acid (oleic acid) contents of milk in dairy sheep (18). In combination with other herbs, turmeric increased fat- and energy-corrected milk yields in cows, while decreasing the acetate-to-propionate ratio in the rumen fluid (19). *Terminalia chebula* and *Phyllanthus emblica* are rich sources of tannins with potential impact on rumen fermentation; particularly a reduction of methanogenesis through decreasing rumen protozoa (20). Supplementation of *T. chebula* at 10 g per kg diet DM in sheep improved nutrient digestibility and fiber degradability possibly through increasing numbers of fibrolytic bacteria (21, 22). Inclusion of *Phyllanthus emblica* has shown to increase *in vitro* dry and organic matter degradability and the synthesis of microbial biomass, while reducing methane production (23). A combination of ajwain, fenugreek, and fennel seeds and fruits of *Terminalia chebula* and *Phyllanthus emblica*, reduced *in vitro* methane production without affecting other fermentation parameters (24). Supplementation of fenugreek led to an improvement *in vitro* dry matter degradability and *in vivo* nutrient digestion and utilization in goats (25, 26). Recently, the inclusion of licorice root has shown to increase protein and saturated fatty acid contents of milk while decreasing unsaturated fatty acids and somatic cell count of goat milk (27).

In present study, we attempted to evaluate the synergistic effects of a mixture of 10 different plant-derived compounds on the rumen bacteriome, rumen fermentation, and milk yield and composition of water buffaloes.

MATERIALS AND METHODS

Animals, Diet, and Experimental Design

This research was carried out at Guangxi Buffalo Research Institute, Nanning, China (latitude 28° 48′N, longitude 108° 22′E). All experimental procedures used in this experiment were approved by the Ethics committee of the Chinese Academy of Agriculture Sciences, Guangxi Buffalo Research Institute, China. Twenty Murrah buffaloes of similar body weight (580 ± 25 kg), parity and stage of lactation (3–4 months) were randomly selected for this experiment and divided into four groups. The four groups of buffaloes were fed with the same basal diet supplemented with 0 (control), 15 (MP15), 25 (MP25), or 35 (MP35) g of a mixture of 10 different phytogenic substances per buffalo per d. Aside from time to exercise and swim, buffaloes were housed individually in an open-sided shed. To exercise, the buffaloes were set free in an adjacent open yard with a stocking density of 15 m^2/buffalo. Free access to water was provided to all buffaloes throughout the day. Fans were installed in the buffalo barn to improve airflow. Buffaloes were allowed 30 min swimming time before milking. Buffaloes were machine milked twice a day. The same experimental diet consisting of maize silage, brewers' grains, and concentrate mixture was fed to all experimental buffaloes for 6 weeks. The buffalo were fed a total mixed ration (TMR) twice per day for *ad libitum* intake. The TMR was formulated to meet the dietary requirements of lactating buffalo. The respective amount of phytogenic supplement was top-dressed on TMR during morning feeding before milking and each buffalo was monitored for leftover. Details of the chemical composition of the experimental diet are given in **Table 1**. The first 2 weeks were considered as an adaptation period. Feed intake of individual buffaloes was measured during the last week of the experiment.

The mixed phytogenic consisted of respective parts of following plants; fennel (seeds), ajwain (seeds), ginger (tubers), *Swertia chirata* (leaves), *Citrullus colocynthis* (fruit), Turmeric, Fenugreek (seeds), *Terminalia chebula* (fruit), Licorice (roots), and *Phyllanthus emblica* (fruit). These plant parts were procured in dry, finely-ground form from Verbena Nutraceuticals Inc.

TABLE 1 | Formulation and chemical composition of the basal experimental diet.

Items	Content
Ingredient (g/kg of DM)	
Corn silage	197
Brewers' grains	418
Concentrate feed mixture*	385
Total	1,000
Chemical composition (g/kg of DM, unless otherwise stated)	
DM (g/kg as fed)	416
OM	756
CP	158
NDF	119
ADF	81
Gross energy (kcal/kg DM)	3.41

Corn 17.83%; wheat bran 7.51%; Soybean meal 5.72%; Lime stone 0.5%; $CaHPO_4$ 0.6%; $NaHCO_3$ 0.8%; NaCl 0.7%; Premix[a] 0.34%.

[a]*The additive premix provided the following per Kg of diets: Vitamin A 550,000 IU, Vitamin E 3,000 IU, Vitamin D3 150,000 IU, 4.0 g Fe (as ferrous sulfate), 1.3 g Cu (as copper sulfate), 3.0 g Mn (as manganese sulfate), 6.0 g Zn (as zinc sulfate), 80 mg Co (as cobalt sulfate).*

(Islamabad, Pakistan). To make up the tested supplement, equal quantities of each compound were thoroughly mixed.

Chemical Composition of the Diet and Mixed Phytogenic

Dry matter (DM), crude protein (CP), and ash content of the feed samples were analyzed according to the standard procedures (28). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using an ANKOM^{2000} Fiber Analyzer (ANKOM Technology Corp., Macedon, NY, USA) including alpha-amylase and sodium sulfite (28, 29). The total polyphenolic content of mixed phytogenic was determined using the Folin-Ciocalteau's phenol reagent as reported previously (30). Gallic acid (10–60 μg/g) was used as standard. The results were expressed as mg of gallic acid equivalent (GAE) per g of MP. Total tannins were measured as tannic acid equivalent and flavonoids were determined as catechin equivalent using UV-VIS Spectrophotometer (Labomed UVD-3500 Spectro) as described previously (31). The chemical composition of the mixed phytogenic is presented in **Table S1**.

Rumen Fermentation Parameters

Rumen content samples (500 ml) were collected only once, at the last day of the experiment before the morning feeding, using a stomach tube. After collection, the samples were directly transported to the laboratory. The rumen pH was measured immediately using a pH meter (HI 9024C; HANNA Instruments, Woonsocket, Rhode Island, USA). Subsequently, the rumen contents were strained through two layers of cheesecloth and subsamples were analyzed for VFA concentrations (C2, C3, C4, C5, iC4, and iC5) using a GC system (Agilent 7890A, Agilent Technologies, USA), as described by Qin (32). A sub-sample of rumen fluid (4 mL) was acidified with 4 mL of HCl (0.2 mol/L) and stored in a freezer (−20°C) for determination of NH_3-N using the indophenols method (33). Microbial protein content was analyzed with a spectrophotometer at 595 nm using 1 mg/ml bovine serum albumin solution (Sigma-Aldrich Co., LLC, St. Louis, Missouri, USA) as standard equivalent (34).

Milk Yield and Composition

Milk yield in the morning (at 5:00 am) and evening (at 5:00 pm) was recorded daily for each buffalo between d 15 and 42. Milk samples for determination of milk composition were collected weekly for 4 consecutive weeks. Fresh milk samples were used to analyze milk composition (milk total solids, protein, fat, and lactose) for morning and evening separately using MilkoScanTM F120 (FOSS, Hillerød, Denmark). Energy corrected milk (ECM) was calculated according to Tyrrell and Reid (35):

$$\text{ECM} = 0.327 \times \text{Milk yield (kg)} + 12.95 \times \text{Fat yield (kg)} + 7.20 \times \text{Protein (kg)}.$$

Determination of Fatty Acid Profile in Milk

Samples from morning and evening milking were pooled (relative to the quantity of milk produced) for each week separately. Milk samples from each week were stored at −20°C until analysis of fatty acids. Briefly, 20 mL of milk was centrifuged in a 50 mL falcon tube at 17,800 × g for 30 min at 4°C. After centrifugation, the above fat layer (1.0 g) was transferred to a 1.5 mL Eppendorf tube and left at room temperature (∼20°C) for ∼20 min to allow fat to melt. After that, it was centrifuged at 19,300 × g for 20 min at room temperature in a microcentrifuge. Centrifugation of fat separated the sample into 3 layers: top layer containing lipid; middle layer containing protein, fat, and other water-insoluble solids; and bottom aqueous layer (36). Milk fatty acids were trans-esterified with sodium methoxide according to Zahran and Tawfeuk (37). Briefly, 2.0 mL of n-hexane were added to 40 ul of butterfat and vortexed for 30 s followed by the addition of 2 mL of sodium methoxide (0.4 mol). After vortexing, the mixture was allowed to settle for 15 min. The upper phase, containing the fatty acid methyl ester (FAME), was recovered and analyzed by an Agilent 7890B Gas chromatography (GC-FID) with a polar capillary column CP-Sil®-88 100 m, 0.25 mm id, 0.2 μm film thickness (Agilent Technologies, USA). Helium was used as a carrier gas at a flow rate of 20 cm s^{-1} and split ratio 100:1. The column temperature profile was held at 100°C for 5 min, ramp to 240°C @ 4°C min^{-1}; hold at 240°C for 30 min. A sample volume of 1.0 μL was injected. The FAME was identified by comparing their relative and absolute retention times with FAME standards (from C4:0 to C22:0). Fatty acid contents are presented as percentage of total fat weight (wt%/wt%).

DNA Extraction and Sequencing of the 16S rRNA Gene

The DNA was extracted from 1 mL of frozen rumen content (both solid and fluid phase) using the CTAB bead-beating method (38). The quality of DNA was checked using a spectrophotometer (NanoDrop2000, Thermo Scientific, USA). High throughput (Illumina MiSeq) sequencing of the 16S rRNA gene was carried out using barcoded primers for V3–V4 region (39). DNA libraries were sequenced using a 2 × 300 paired-end sequencing module (Illumina, San Diego). Resultant paired-end sequence reads were joined

TABLE 2 | Effect of mixed phytogenic on rumen fermentation parameters.

Item	Treatments				SEM	*P*-value
	Control	MP15	MP25	MP35		
pH	6.68[b]	6.88[a]	6.79[ab]	6.81[a]	0.026	0.029
TVFAs (mmol/L)	34.87	31.07	31.34	32.87	1.027	0.586
Acetate (mmol/L)	17.54	16.07	16.50	16.95	0.446	0.771
Propionate (mmol/L)	9.99	8.43	8.52	8.99	0.353	0.408
Isobutyrate (mmol/L)	0.63	0.69	0.63	0.65	0.017	0.714
Butyrate (mmol/L)	5.55	4.79	4.69	5.20	0.240	0.611
Isovalerate (mmol/L)	0.68	0.69	0.61	0.67	0.036	0.884
Valerate (mmol/L)	0.45	0.39	0.37	0.40	0.025	0.699
Acetate/Propionate	1.78	1.91	1.93	1.89	0.039	0.559
MCP (mg/mL)	37.93	42.23	43.72	38.73	1.418	0.436
NH_3-N (mg/mL)	11.94	10.66	9.91	10.24	0.837	0.857

MP15, mixed phytogenic fed @ 15 g/d/head; MP25, mixed phytogenic fed @ 25 g/d/head; MP35, mixed phytogenic fed @ 35 g/d/head; Control, without mixed phytogenic; TVFAs, Total volatile fatty acids; MCP, Microbial crude protein; NH_3-N, Ammonia Nitrogen. [a,b] Values with different superscripts in the same row differ significantly ($P < 0.05$).

TABLE 3 | Effect of mixed phytogenic on milk yield parameters.

Parameter	Control	MP15	MP25	MP35	SEM	*P*-value
Dry matter intake (kg/d)	7.78	8.16	8.06	8.26	0.109	0.479
Milk yield (kg/d)	8.69	8.52	8.50	8.57	0.554	1.000
Fat corrected milk (kg/d)	13.36	14.03	13.07	14.61	0.740	0.907
Energy corrected milk (kg/d)	14.45	15.04	14.01	15.49	0.805	0.937
Protein (%)	4.99	5.00	4.65	4.89	0.064	0.181
Protein yield (kg/d)	0.44	0.43	0.39	0.42	0.028	0.965
Fat (%)	7.83	8.28	7.73	8.69	0.248	0.539
Fat yield (kg/d)	0.65	0.71	0.64	0.74	0.034	0.339
Total solids (%)	19.25	19.56	18.70	19.84	0.280	0.562
Solid not fat (%)	10.70	10.53	10.32	10.33	0.072	0.194
Lactose (%)	5.32	5.21	5.42	5.21	0.037	0.108

MP15, mixed phytogenic fed @ 15 g/d/head; MP25, mixed phytogenic fed @ 25 g/d/head; MP35, mixed phytogenic fed @ 35 g/d/head; Control, without mixed phytogenic.

together using their overlap relationship (minimum 10 bp) allowing maximum mismatch ratio of 0.2 using FLASH and Trimmomatic software. After pruning, optimized sequence reads were aligned against the SILVA database, Release128 (http://www.arb-silva.de) for identification of Operational Taxonomic Units (OTU) using cluster identity threshold of 97% (40, 41). After that taxonomy of each sequence (OTU representative) was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the database (confidence threshold of 0.7). Taxonomic assignment of rumen bacteria was performed with bioinformatics pipeline of Qiime software (http://qiime.org/scripts/assign_taxonomy.html) as described previously (42).

The bacterial diversity of treatment groups was determined by analyzing alpha and beta diversity indices. Population richness (Chao, ACE) and evenness (Shannoneven and Simpsoneven) of rumen bacteria were analyzed for each sample (43). Alpha diversity was estimated by determining Shannon and Simpson indices (44–47). Beta diversity index was calculated to analyze rumen bacterial diversity across different treatment groups using Bray-Curtis dissimilarities (48). Bray-Curtis dissimilarities among different treatment groups were evaluated non-parametrically by utilizing permutation analysis of variance method (PERMANOVA using 999 permutations) as previously reported (49). Redundancy analysis (RDA) was performed at the bacterial genus level using VFAs and milk yield parameters as explanatory variables in the vegan R package (version 3.2).

Statistical Analysis

Effect of MP on all parameters related to milk yield and composition; DM intake, rumen fermentation, and bacterial alpha diversity were analyzed using the general linear model in SAS (SAS Institute Inc., Cary, NC, USA) with treatment as a fixed effect and buffalo as a random effect nested in treatment group. The Duncan's multiple range test was used as a *post-hoc* test to identify differences among treatment groups. We also analyzed three orthogonal contrasts including all MP treatments vs. the control, linear effect of MP dose, and quadratic effect of MP dose. Treatment effects were declared significant at $P < 0.05$ and trends were discussed at $0.05 \leq P < 0.1$. The abundances of bacterial phyla and genera were compared using the Kruskal-Wallis H test with a false discovery rate (FDR) correction and Scheffer as a *post-hoc* test to elucidate differences across treatment groups.

Spearman's rank correlation (r) analyses were performed with the vegan R package (version 3.2) to analyze the association of relative abundance of bacterial genera with rumen fermentation and milk yield parameters. Correlation heatmaps were constructed using the corrplot R package. In the two-dimensional heat map, change in defined color and its depth indicates the nature and strength of the correlation, respectively. Asterisk sign was used when the r value was >0.1 and the *P*-values were <0.05 ($^{*}0.01 < P \leq 0.05$, $^{**}0.001 < P \leq 0.01$, $^{***}P \leq 0.001$).

RESULTS

Rumen Fermentation Parameters

Supplementation of MP increased ruminal pH ($P = 0.029$) in MP15 and MP35 but no change in pH was observed in MP25 compared to the control (**Table 2**). There was no effect of treatment on any other rumen fermentation parameter.

Dry Matter Intake (DMI), Milk Yield, and Composition

There was no treatment effect on DMI and milk production performance (**Table 3**).

Fatty Acids Composition of Milk

Supplementation of MP increased ($P < 0.05$) short-chain fatty acids in MP15 compared to other groups (**Table 4**). Myristic acid (C14:0) tended to increase, while C18:0 tended to decrease in MP15 as compared to other groups. The C16:1 content and its unsaturation index was higher ($P < 0.05$) in MP35 as compared to the control and other treatment groups. Furthermore, C18:3n3 was ($P < 0.05$) higher in control, MP15 and MP25, as compared

TABLE 4 | Milk fatty acids profile across different treatment groups.

Fatty acid	Common name	Control	MP15	MP25	MP35	SEM	*P*-value
C4:0	Butyric acid	0.86^{ab}	0.95^{a}	0.83^{bc}	0.74^{c}	0.035	0.001
C6:0	Caproic acid	1.03^{ab}	1.12^{a}	0.96^{b}	1.05^{ab}	0.019	0.023
C8:0	Caprylic acid	0.68^{ab}	0.78^{a}	0.62^{b}	0.73^{a}	0.018	0.014
C10:0	Capric acid	1.49^{b}	1.88^{a}	1.39^{b}	1.64^{ab}	0.053	0.014
C12:0	Lauric acid	2.19^{b}	2.63^{a}	2.03^{b}	2.32^{ab}	0.066	0.016
C14:0	Myristic acid	10.64^{b}	11.55^{a}	10.45^{b}	11.03^{ab}	0.161	0.054
C14:1	Myristoleic acid	1.04	1.11	1.10	1.14	0.016	0.125
C16:0	Palmitic acid	31.91	32.22	32.76	33.19	0.366	0.671
C16:1	Palmitoleic acid	1.88^{b}	1.77^{b}	1.90^{b}	2.19^{a}	0.044	0.001
C17:0	Margaric acid	0.3	0.32	0.30	0.28	0.009	0.474
C18:0	Stearic acid	15.97^{a}	14.50^{b}	15.41^{ab}	14.63^{ab}	0.267	0.086
C18:1	Oleic acid	28.87	27.69	28.89	27.78	0.354	0.488
C18:2n6	Linoleic acid	1.42	1.56	1.53	1.43	0.029	0.255
C18:3n3	α-Linolenic acid	0.43^{ab}	0.48^{a}	0.47^{a}	0.39^{b}	0.014	0.035
C18:3	Linolenic acid	1.51	1.40	1.47	1.51	0.042	0.639
Group of fatty acids, g/100 g of fatty acids							
SFA		64.82	65.97	64.65	65.53	0.393	0.617
UFA		35.17	34.02	35.34	34.46	0.393	0.617
MUFA		31.81	30.59	31.89	31.12	0.357	0.554
PUFA		3.36	3.43	3.45	3.34	0.060	0.891
SCFA		3.98^{b}	4.75^{a}	3.71^{b}	4.12^{b}	0.097	0.001
MCFA		47.51	49.31	48.26	49.83	0.504	0.365
LCFA		48.51	45.94	48.03	46.05	0.553	0.224
n-6/n-3		3.35	3.47	3.43	3.86	0.128	0.419
Unsaturation index, %							
C14:1/(C14:0 + C14:1)		9.19	8.84	9.63	9.42	0.144	0.283
C16:1/(C16:0 + C16:1)		5.61^{b}	5.25^{b}	5.49^{b}	6.25^{a}	0.125	0.01
C18:1/(C18:0 + C18:1)		64.41	65.9	65.18	65.44	0.387	0.427

SFA, Saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, poly unsaturated fatty acids; SCFA, short-chain fatty acids; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acids; SCFA included the C4:0, C6:0, C8:0, and C10:0 fatty acids; MCFA included all linear fatty acids from C12:0 to C16:1; LCFA included all linear fatty acids from C17:0 to C18:3. MP15, mixed phytogenic fed @ 15 g/d/head; MP25, mixed phytogenic fed @ 25 g/d/head; MP35, mixed phytogenic fed @ 35 g/d/head; Control, without mixed phytogenic. ^{a,b,c} *Values with different superscripts in the same row differ significantly ($P < 0.05$).*

to MP35. There was no treatment effect on total UFA, MUFA, and PUFA content as well as omega6 to omega3 ratio.

Rumen Bacterial Diversity

High throughput sequencing of the 16S rRNA gene revealed a total of 2,780 OTU in the rumen content samples. The distribution of shared and unique OTU of the four treatment groups is presented in **Figure 1**. The highest number of OTU was detected in buffaloes supplemented with MP25, compared to control and other groups. The number of OTU increased in response to MP15 and MP25 but decreased for MP35 as compared to the control. A total of 1,413 OTU were shared by all groups, while the number of unique OTU was 536. The highest number of unique OTU was found in MP15 (163) followed by MP25 (161), MP35 (123), and the control (50).

Treatment had no effects on alpha diversity parameters (**Table 5**). Analysis of beta diversity showed the difference between groups caused by dietary treatment. The first two dimensions from the (non-metric) multi-dimensional scaling (NDMS) of the Bray-Curtis dissimilarity matrix are presented in **Figure 2**. Samples were grouped by the level of MP and PERMANOVA (using 999 permutations) amongst all groups showed effect of treatment ($P = 0.025$).

Relative Abundance of Rumen Bacteria

Bacteroidetes and *Firmicutes* were the most dominant phyla representing between 85 and 91% of total bacteria detected in the rumen of the buffaloes (**Figure 3**). The relative abundance of *Firmicutes* and *Proteobacteria* increased while *Bacteroidetes* and *Spirochaetes* decreased numerically in response to treatment compared to the control (**Table S2**). The abundance of *Cyanobacteria* increased in MP15 and MP35 but decreased numerically in MP25 as compared to the control group.

Prevotella was the dominant genus in all four treatments, representing about 31–49% of all sequences (**Table S3**). Relative abundance of *Prevotella* decreased numerically with increasing levels of MP (**Figure 4**). Second most abundant genus was *o-Clostridales*, which increased with supplementation particularly in MP15 (5.74%) and MP25 (5.53%) as compared to MP35 (3.62%) and control (4.96%). Abundance of *f__F082*

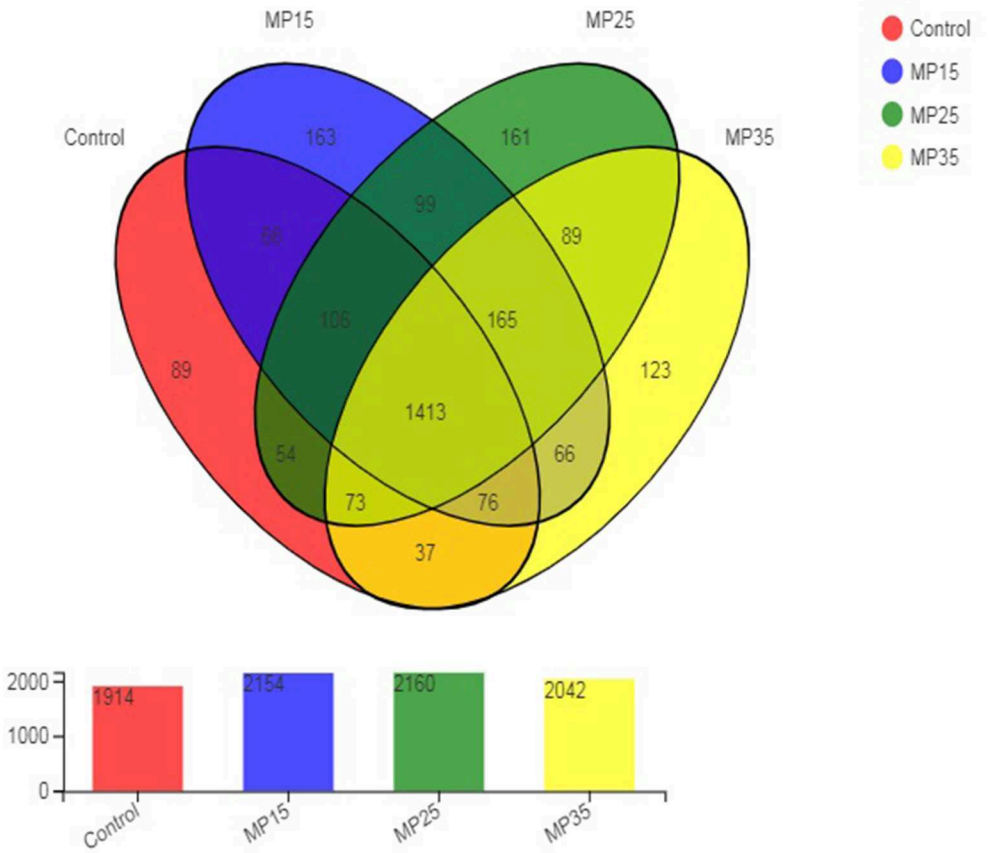

FIGURE 1 | Distribution of OTU across different treatment groups.

increased in MP25 (4.01%) and MP35 (4.40%) compared to MP15 (3.09%) and control (3.45%). Similarly, abundance of *Rikenellaceae_RC9_gut*_group also increased in MP25 (3.95%) and MP35 (4.01%) compared to MP15 (2.99%) and the control (3.09%). *Treponema* decreased linearly in response to MP supplementation. Highest relative abundance of *Christensenellaceae_R-7*_group was observed in MP25 (2.39%) and MP35 (2.33%) as compared to MP15 (1.80%) and control group (2.07%). Reduced abundance of *Succiniclasticum* and *Prevotellaceae_UCG-003* was observed in MP-supplemented buffaloes compared to the control. Relative abundance of *Butyrivibrio* increased with supplementation of MP, particularly in MP35, compared to the control. The relative abundance of *Ruminococcaceae* also increased as result of MP supplementation. *Pseudobutyrivibrio* decreased in response to MP15 but was present in greater abundance in MP25 and MP35 compared to the control. The relative abundance of *Succinibrionaceae_UCG-002* increased in MP35 (2.33%) compared to MP15 (0.66%), MP25 (0.41%), and the control (1.17%).

Association of Bacteria With Rumen Fermentation Parameters

Redundancy analysis showed acetate contributed to the bacterial community differences at genus level (contribution = 56.8%, $P = 0.023$) among the four treatment groups (**Figure 5**). Milk yield and composition parameters did not contributed to overall differences in bacterial genera. Two bacterial genera *Prevotella_1* (contribution = 78.6, $P = 0.04$) and *Treponema_2* (contribution = 13.3, $P = 0.045$) contributed substantially to the compositional differences in rumen bacteriome (**Figure 5**).

Spearman's correlation between the relative abundance of bacterial genera and rumen fermentation parameters is shown in **Figure 6**. Acetate concentrations were negatively correlated with *Treponema_2* ($R = -0.59$; $P < 0.05$), *Fibrobacter* ($R = -0.66$;

TABLE 5 | Effect of mixed phytogenic on bacterial alpha diversity parameters.

Items	Control	MP15	MP25	MP35	*P*-value
Shannon	5.619	5.759	5.854	5.908	0.095
Simpson	0.013	0.012	0.010	0.008	0.231
ace	1892.8	1999.3	1956.7	1930.4	0.620
Chao	1777.1	2001.4	1955.7	1978.1	0.102
Shannoneven	0.785	0.787	0.800	0.814	0.260
Simpsoneven	0.063	0.057	0.069	0.093	0.451

MP15, mixed phytogenicfed @ 15 g/d/head; MP25, mixed phytogenic fed @ 25 g/d/head; MP35, mixed phytogenicfed @ 35 g/d/head; Control, without mixed phytogenic.

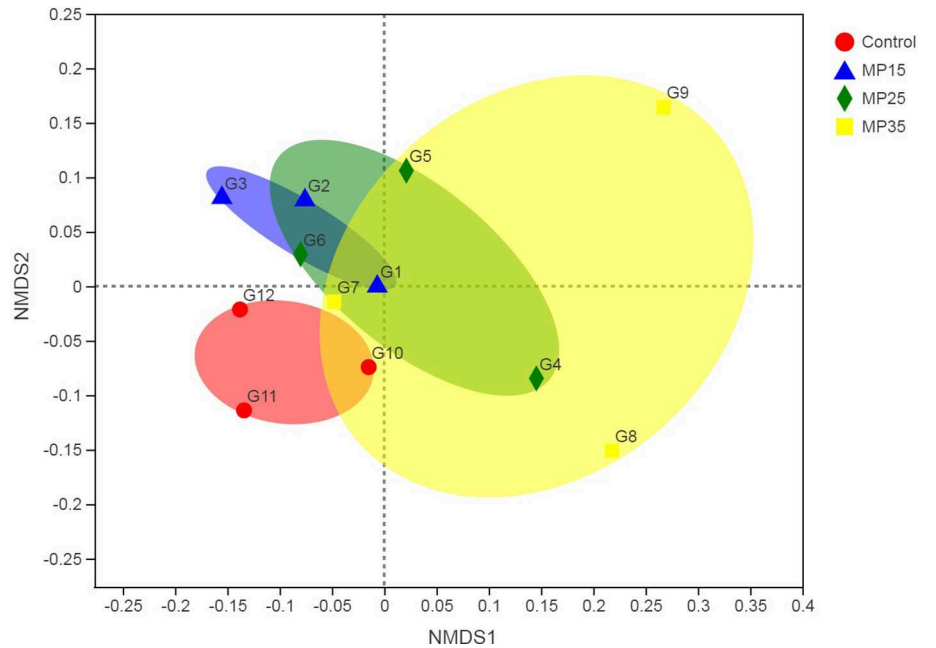

FIGURE 2 | First two dimensions from the (non-metric) multi-dimensional scaling of the Bray-Curtis dissimilarity matrix. Samples were grouped by feed additive. PERMANOVA amongst all groups $p = 0.025$ (using 999 permutations).

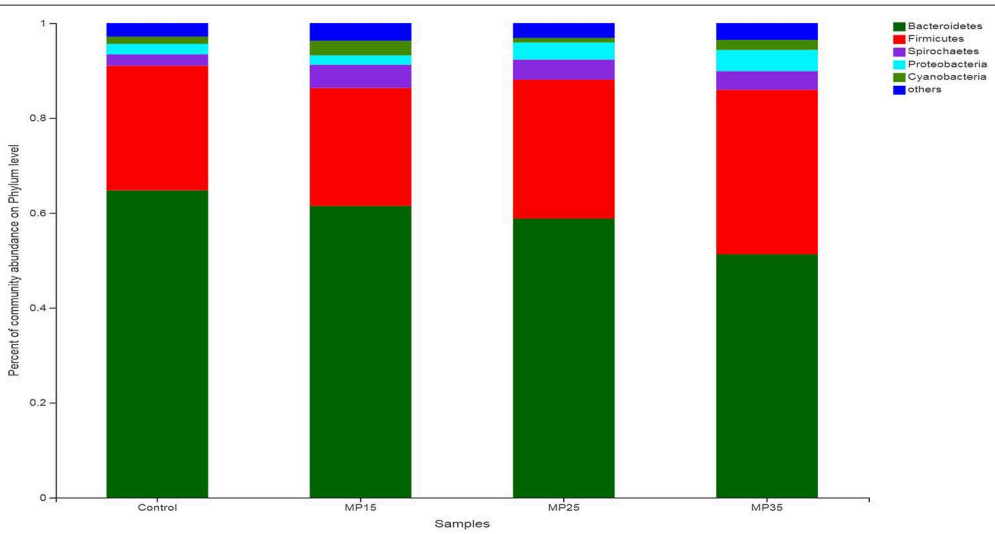

FIGURE 3 | Relative abundance of bacterial phyla across treatment groups.

$P < 0.05$), and *Candidatus_Saccharimonas* ($R = -0.64$; $P < 0.05$). Propionate, butyrate and valerate were negatively correlated with genus *f_F082*, *Rikenellaceae_RC9_gut_group*, *Prevotellaceae_UCG-001* ($R = -0.75$, $P < 0.01$, *Lachnospiraceae_AC2044_group*, *Ruminococcaceae_UCG-005*, *Lachnospiraceae_ND3007_group*, and *probable_genus_10*, while two genera *Ruminococcaceae_NK4A214_group* and *Candidatus_Saccharimonas* were negatively correlated with propionate but not with butyrate (**Table S4**). Isobutyrate showed a positive correlation ($R = 0.60$; $P < 0.05$) with genus *Succiniclasticum*, while isovalerate was negatively correlated ($R = -0.59$; $P < 0.05$) with *Ruminococcaceae_UCG-005*. Only one bacterial genus *f_Muribaculaceae* showed positive correlation ($R = 0.71$; $P < 0.05$) with acetate to propionate ratio. TVFAs showed a negative correlation with genus *f_F082*, *Rikenellaceae_RC9_gut_group*, *Ruminococcaceae_UCG-005*, and *Candidatus_Saccharimonas*. Three bacterial genera *Rikenellaceae_RC9_gut_group*, *f_Prevotellaceae*, and *Fibrobacter*

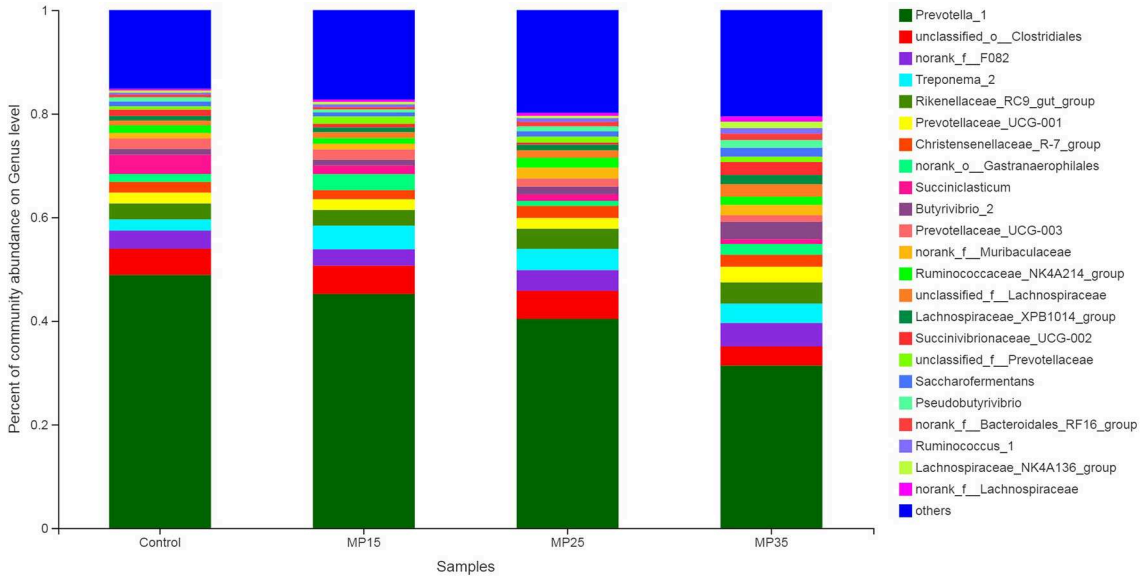

FIGURE 4 | Relative abundance of bacterial genera across treatment groups.

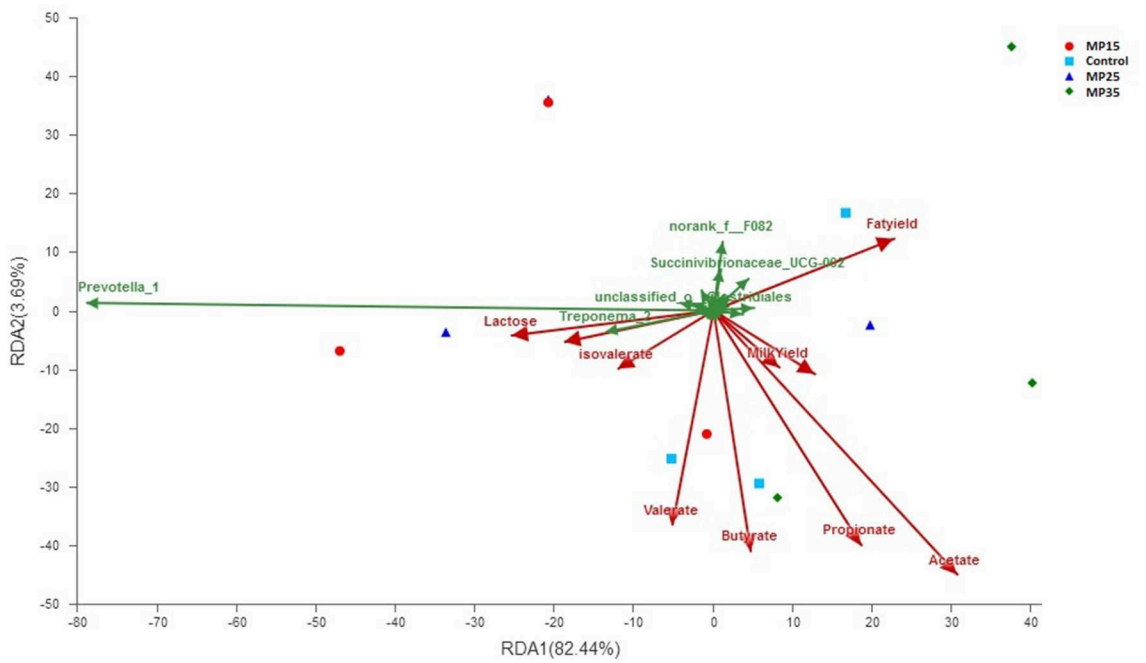

FIGURE 5 | Biplot of RDA analysis on genus level between bacterial genera, VFA, and milk yield parameters.

were positively correlated with the ruminal concentration of NH_3-N.

Association of Rumen Bacteria With DMI, Milk Yield, and Composition

Milk yield was positively correlated with genera *o_Clostridiales* ($R = 0.59$; $P < 0.05$), *Butyrivibrio_2* ($R = 0.59$; $P < 0.05$), *Pseudobutyrivibrio* ($R = 0.67$; $P < 0.05$), and *Lachnospiraceae_NK3A20_group* ($R = 0.58$; $P < 0.05$; **Figure 7**, **Table S5**). A moderate negative ($R = -0.61$; $P < 0.01$) correlation of milk yield was observed with *Prevotellaceae_UCG-003*. Milk protein contents were negatively ($R = -0.64$; $P < 0.05$) correlated with *Ruminococcaceae_NK4A214_group* while milk protein yield was positively correlated ($R = 0.61$; $P < 0.05$) with

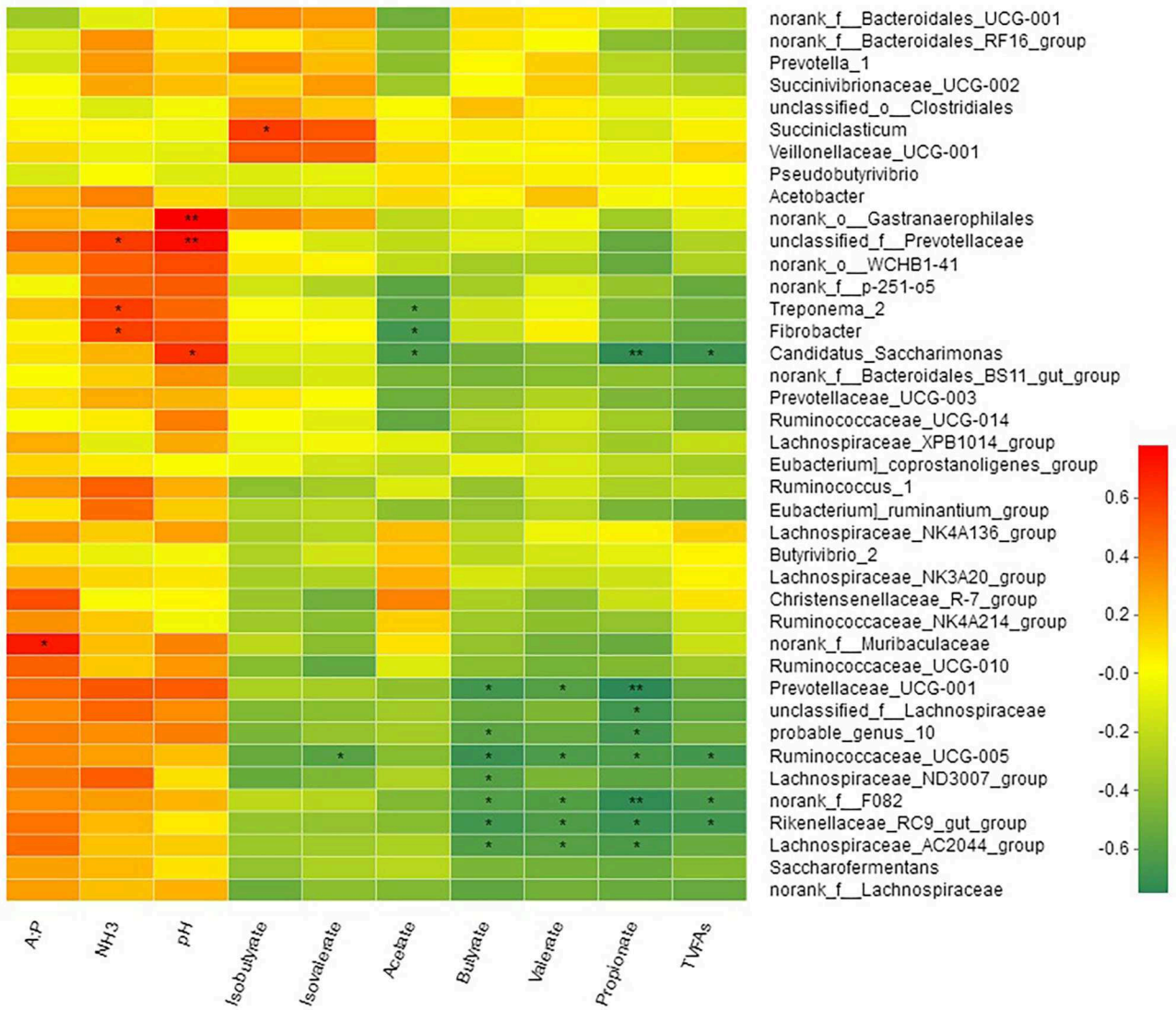

FIGURE 6 | Correlation of bacterial genera with rumen fermentation parameters.

Pseudobutyrivibrio. Milk fat yield showed positive correlation with; *o_Clostridiales* ($R = 0.56$; $P < 0.05$), *Butyrivibrio_2* ($R = 0.69$; $P < 0.05$), *Lachnospiraceae_XPB1014_group* ($R = 0.70$; $P < 0.05$), *Pseudobutyrivibrio* ($R = 0.68$; $P < 0.05$), and *Lachnospiraceae_NK3A20_group* ($R = 0.64$; $P < 0.05$). Milk lactose was negatively ($R = -0.78$) correlated with *Lachnospiraceae_XPB1014_group*. Bacteria specialized in fiber and polysaccharides degradation including *Succinivibrionaceae_UCG-002* ($R = 0.63$; $P < 0.05$), *Ruminococcus_1* ($R = 0.65$; $P < 0.05$), *Lachnospiraceae_NK4A136_group* ($R = 0.60$; $P < 0.05$), *Fibrobacter* ($R = 0.65$; $P < 0.05$), and *Acetobacter* ($R = 0.62$; $P < 0.05$) were positively correlated with DMI (**Figure 7**).

DISCUSSION

Rumen Fermentation Parameters

The mixed phytogenic tested in this study had no effect on rumen fermentation parameters of buffaloes except pH. An increase in rumen pH in response to supplementation of phytochemicals (flavonoids and polyphenols) in ruminants has been reported earlier (51, 52). A stabilization of rumen pH and prevention of acidotic bouts would be particularly beneficial for ruminants fed large amount of readily fermentable carbohydrates which can decrease rumen pH rapidly and alter fermentation kinetics and the composition of rumen microbiome. Strong declines in rumen pH reduce the activity of cellulolytic bacteria (53), shift bacterial populations and promote lysis of gram-negative bacteria

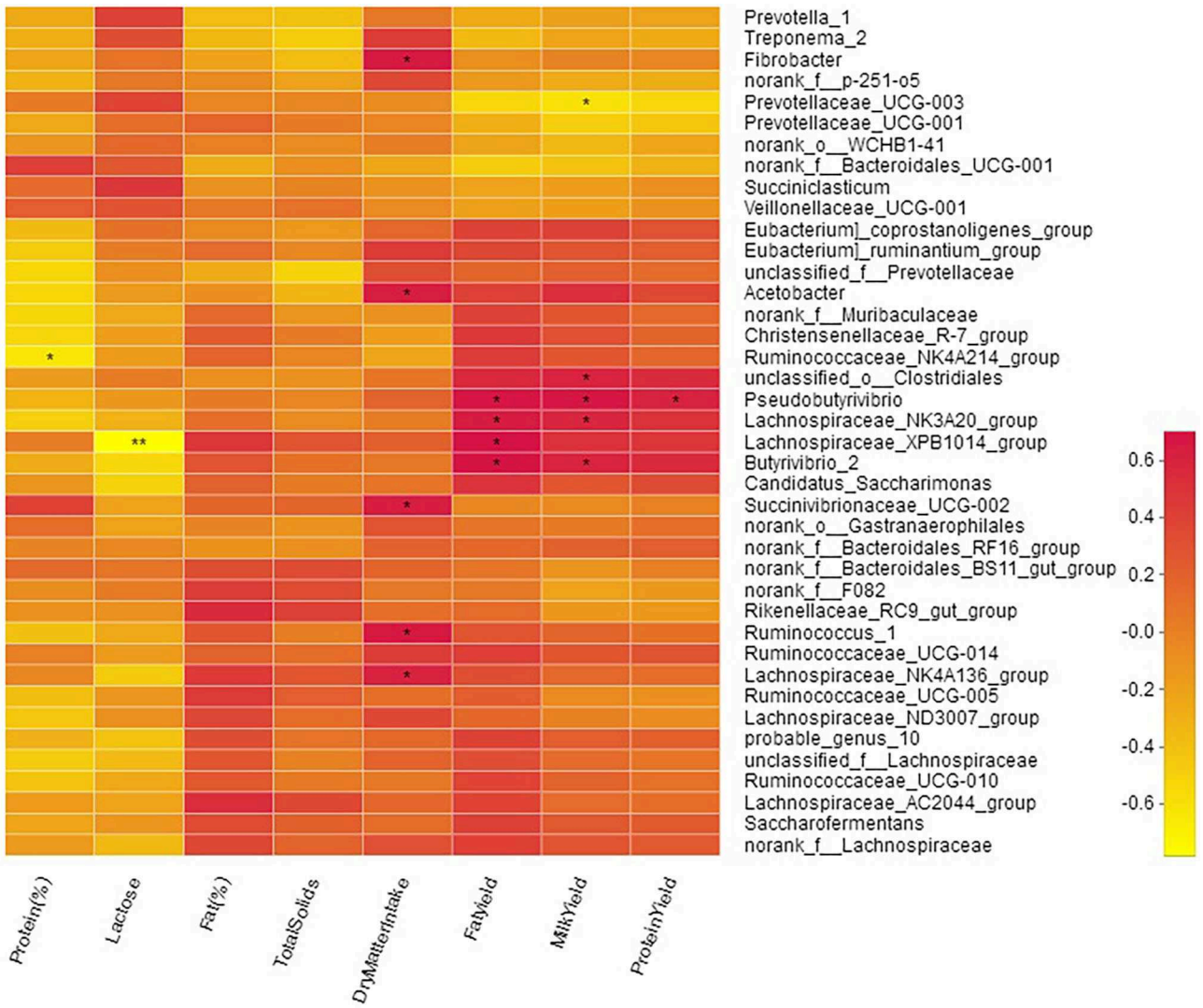

FIGURE 7 | Correlation of bacterial genera with milk yield parameters.

leading to an increase in lipopolysaccharides (LPS) in the rumen (54, 55). Plant polyphenols have shown to increase rumen pH and stimulate the diversity of rumen microbiota, which is commonly high in conditions of physiological rumen pH and regular rumen function (56).

Rumen pH plays a crucial role in fiber degradation as it directly affects bacterial adhesion to cellulosic material (57, 58). This can lead to a reduction in fiber digestion, as frequently observed in animals fed high-grain diets (59). In addition, fibrolytic bacteria like *Rumiococcus* and *F. Succinogenes* are highly sensitive to even mildly acidic pH (60). Our findings indicate that the tested combination of phytogenics might improve the performance and health of ruminants by preventing excessive drop in pH and subsequent accumulation of LPS in rumen. However, it needs to be emphasized that we measured rumen pH only once as spot sampling just before the morning feeding. It may not be reflective of pH changes over the course of the day, which are important variables to consider. Earlier studies have reported the strong diurnal variation of rumen fermentation parameters and particularly pH (61). The limited number of buffaloes enrolled in the experiment and the fact that we only sampled each buffalo once, is a limitation of our study. In addition, we also did not collect rumen samples at the beginning of the experiment but considered the control group as baseline for our experiment. A more extensive rumen sampling protocol should be followed in further experiments.

We observed an increase in the relative abundance of well-known bacterial genera like *Pseudobutyrivibrio, Butyrivibrio*, and *Succinivibrioanceae*. These bacteria form butyrate and propionate which can subsequently affect milk composition especially the milk fat content. The shift in the relative abundance of certain bacterial genera had, besides the discussed modulation

of rumen pH, no other effect on rumen fermentation. This might be due to the functional redundancy of the rumen microbiota. The rumen microbiome possesses the ability to adapt to long term exposure to inhibitory substances, like some phytogenics but the effectiveness of the adaptation is dependent on the robustness and diversity of the microbiome, length of exposure, and the concentration of the inhibitor (62). No change in rumen fermentation parameters in response to phytogenic compounds like peppermint oil, garlic, and *Piper sarmentosum* was reported earlier (63, 64).

DMI, Milk Yield, and Composition

Earlier studies have also reported no effect of plant compounds, such as propolis polyphenols, garlic and peppermint on DMI and the apparent digestibility of nutrients in buffaloes (63, 65). As it was the case in the present study, other studies also reported that polyphenolic compounds had no negative impact on milk yield. For example, supplementation of propolis polyphenols had no effect on milk yield and concentration of milk solids in dairy cows (66). Studies using a blend of different phytochemicals like cinnamaldehyde, eugenol and capsicum also reported no effects on milk yield in dairy cattle (67–69).

Milk Fatty Acid Contents

The major milk fatty acids were C16:0 and C18:1, followed by C18:0 and C14:0, which is in agreement with earlier studies in dairy cattle (70, 71). Contents of SFA (65%) and UFA (35%) measured in our study are similar to earlier reports in cattle and buffaloes (72, 73).

Supplementation of MP15 increased the content of short-chain fatty acids (C4 to C10:0) in milk. The increase in C18:3n3 in response to MP15 and MP25 means that MP has the potential to affect de novo synthesis of fatty acids. The tendency to decrease the percentage of stearic acid (C18:0), a major saturated fatty acid, is desirable from a human health point of view. Polyphenolic compounds have shown to affect microbial biohydrogenation by inhibiting specific rumen bacteria, this can lead to a more desirable fatty acid composition of milk (74–76). Condensed tannins have shown to partially inhibit the last step of C18:3 biohydrogenation in the RUSITEC system (77). Durmic et al. (78) reported that tannins extracted from *Acacia mearnsii* inhibited *Clostridium proteoclasticum* but exhibited no effect on *Butyrivibrio fibrisolven,* revealing selective inhibition of rumen bacteria involved in biohydrogenation.

Earlier studies reported that polyphenolic-rich forage increased the α-linoleic acid content of milk in sheep (79, 80). Higher abundance of *Butyrivibrio* and *Pseudobutyrivibrio* was associated with an increase in the content of unsaturated fatty acids owing to their positive correlation with linoleic acid and n-3 fatty acid content of milk (81). The decrease in stearic acid (C18:0) together with the increase in n-3 fatty acid contents, is in agreement with earlier studies that reported similar findings in response to supplementation of tannins in dairy sheep (82). Based on the ratio of C14:1 to C14:0 (a proxy of desaturation), it has been suggested that tannins can enhance the activity of stearoyl Co-A desaturase enzyme (SCD), which mediates the conversion of stearic acid to oleic acid and vaccenic acid to conjugated linolenic acid (CLA). In particular, SCD has shown to contribute almost 50% of oleic acid and cis-9, trans-11 CLA secreted in sheep milk (83). This implies that tannins can increase milk unsaturated fatty acids especially n-3 fatty acids not only by mediating rumen biohydrogenation but also through enhancing SCD activity (82, 84, 85).

Since we did not determine the fatty acids content of the rumen microbial biomass, we are unable to associate microbial abundance with the fatty acid profile in milk. This should be attempted in future studies.

Rumen Bacterial Diversity

Supplementation of MP had no effect on bacterial alpha diversity. However, beta diversity was impacted by MP. Similar results regarding alpha and beta diversity have been reported earlier in response to grape-pomace which is rich in polyphenols (86).

As it was the case in this study, *Bacteroidetes* and *Firmicutes* are the major bacterial phyla in both dairy cattle and buffaloes (50, 87–90). A linear increase in *Firmicutes* was observed together with a decrease in *Bacteroidetes*. The highest increase in relative abundance in *Firmicutes* was observed in response to the highest dose of phytogenics (MP35) and resulted in a corresponding decrease in *Bacteroidetes*. An increase in *Firmicutes-to-Bacteroidetes* ratio in response to supplementation of plant flavonoids has been reported earlier (50). A major function of rumen *Bacteroidetes* is the breakdown of polysaccharide, along with various other activities (91). *Firmicutes* are particle-associated bacteria, which produce butyrate. Numerically higher concentration of butyrate in MP35 was associated with a greater abundance of *Firmicutes*. Furthermore, buffaloes in this group also had higher milk fat percentage and fat yield, likely due to the positive association of *Firmicutes-to-Bacteroidetes* ratio and milk fat yield, as previously reported (92).

In the present study, *Prevotella* was the dominant genus across all treatment groups. This is in agreement with earlier studies in buffalo (93–95). Supplementation of MP linearly decreased the abundance of *Prevotella*, with the greatest reduction (1.6-fold) in MP35 compared to the control. The decrease in *Prevotella* was correlated with numerically higher DMI, fat corrected milk (FCM), ECM, fat (%), and milk fat yield in MP35 as compared to the control, most likely due to the negative association of *Prevotella* with DMI and milk fat content (92, 96). We also observed a negative correlation of *Prevotella* with acetate, propionate, milk yield and fat (%) but these correlations were weak ($R = 0.2–0.37$) and not significant. In contrast, earlier studies have also reported a positive correlation of *Prevotella* with acetate and butyrate in dairy cows (97, 98) and butyrate in buffaloes (99). *Prevotella* species are more specialized in protein degradation, peptide fermentation and their uptake in the rumen (100). Similar to *Prevotella*, lower abundance of *Succiniclasticum* and *Prevotellaceae*_UCG-003 was observed in buffaloes supplemented with MP compared to the control. In response to MP35, we detected a 3-fold increase in the relative

abundance of *Butyrivibrio,* together with a 1.8-fold increase in *Pseudobutyrivibrio* compared to the control. Bacterial taxa like *Firmicutes, Butyrivibrio,* and *Pseudobutyrivibrio,* are important degraders of polysaccharides in the rumen and produce formate, butyrate, and acetate (101).

Plant phenolic compounds like thymol; have shown to increase the relative abundance of *Firmicutes in vitro* (up to 82.8%) mainly by inhibiting more sensitive non-*Firmicutes* (*Bacteroidetes*) bacteria (102). In contrast, plant essentials oils extracted from *Origanum vulgare*, garlic and peppermint have shown to decrease the abundance of *Firmicutes* and methane production, while increasing *Bacteroidetes* (7). The increase in *Proteobacteria* in response to MP supplementation was interesting as a substantial increase (2-fold) in the relative abundance of *Succinibrionaceae* in response to MP35 was also observed. Previously, plant secondary metabolites (8-hydroxyquinoline, α-terpineol, camphor, bornyl acetate, α-pinene, thymoquinone, and thymol) have shown to increase the relative abundance of *Succinibrionaceae* (102). In study *Succinibrionaceae* was a dominant family of *Proteobacteria*, which is in agreement with earlier data from cattle (103). The major fermentation product of this bacterial family is succinate which is subsequently converted to propionate in the rumen, so it creates the possibility of competition between *Succinivibrioanceae* and methanogens to utilize hydrogen as a substrate to produce succinate and propionate instead of methane. In line with this, greater abundance of *Succinivibrioanceae* was negatively correlated with methane production ($R = -0.72$) in cattle (102). Substantially higher abundance of *Succinivibrioanceae* has been observed in beef cattle with low methane production compared to cattle with higher emissions (103). In addition to the fact that methane is a strong greenhouse gas, reduced losses of methane can also be associated with an improvement in feed efficiency in ruminants. Since we did not measure methane production or total methanogens, we can only speculate about the effect of MP on methane emissions. However, the detected shift in rumen bacteriome toward more beneficial bacteria like *Pseudobutyrivibrio, Butyrivibrio* and *Succinivibrioanceae* make it somewhat likely that the tested phytogenic has not only positive impact on production performance but also greenhouse gas intensity of milk.

Association of Bacteria With Rumen Fermentation and Milk Yield Parameters

The Spearman's correlation analysis revealed 28 negative and 8 positive correlations of bacterial genera with rumen fermentation parameters. Three bacterial genera *Fibrobactor, Treponema_2*, and *f_Prevotellaceae* had a modest positive correlation with ruminal NH_3-N concentrations. *Treponema* belongs to phylum *Spirochetes* which mostly ferments soluble sugars to formic acid, acetic acid, lactic acid, and succinic acid (104). *Succiniclasticum* was positively correlated with the concentration of isobutyrate in the rumen as this genus of bacteria is associated with the formation of succinate from starch degradation leading to the subsequent production of *propionate* (105). Moreover, increased abundance of *Succiniclasticum* in high-producing dairy cows has been associated with greater propionate production (106). In our study, we observed very few positive correlations in contrary to earlier studies reporting various strong correlations of bacterial genera with VFA in the rumen of dairy cows (81, 107) and buffaloes (99). This may be attributed to the low variation observed in fermentation parameters, which was likely due to the relatively low sample size and the fact that we only sampled once instead of multiple times over the course of the day. We took rumen samples once from each buffalo using the stomach tube at the end of the experiment; consequently we had a total of five samples per treatment. The relatively low number of buffaloes per treatment and only one rumen sampling are the main limitations of the present study. To evaluate potential effects of MP on rumen fermentation and shifts in the bacterial population in more detail, further studies are required involving a larger cohort of animals and multiple rumen samplings.

Fibrobactor is one of the most active cellulolytic bacteria which ferment only cellulose, glucose, and cellobiose, its primary end products are acetic and succinic acid (104). Unsurprisingly, in this study presence of Fibrobactor was positively correlated with DMI due to its ability to breakdown fiber. However, the negative correlation between *Fibrobactor* and acetate is difficult to explain. All five bacterial genera (*Succinivibrio, Ruminococcus, Lachnospiraceae, Fibrobacter, and Acetobacter*) which were positively correlated with DMI are well-known cellulolytic and amylolytic bacteria (108). Dry matter intake has a direct association with milk production so the relationship of these bacteria with DMI, as observed in this study, indicates their potential in enhancing milk yield in buffaloes (109).

Our study showed a positive correlation of *Pseudobutyrivibrio* with milk, fat and protein yield. The positive impact of *Pseudobutyrivibrio* on milk yield parameters has been reported earlier in dairy cows (108). A positive correlation of *Butyrivibrio* and *Lachnospiraceae* with milk yield and protein has also been reported (93). Furthermore, a positive correlation of *Butyrivibrio* with average milk fat, milk solid, and total milk yield has been reported in buffaloes (99). *Butyrivibrio* and *Pseudobutyrivibrio* ferment structural carbohydrates (hemicellulose, xylan, and pectin) to butyrate (110). However, a negative correlation of *Butyrivibrio* species with milk fat yield has also been reported in dairy cattle (81, 96). The substantial increase in the relative abundance of *Butyrivibrio* (3-fold) and *Pseudobutyrivibrio* (1.8-fold) in this study was correlated with numerically higher milk fat (%), DMI, FCM, ECM, and DMI in buffaloes supplemented with MP35. An unclassified genus of family *Prevotellaceae* showed a negative correlation with milk yield which is also in agreement with earlier reports revealing a negative association of *Prevotella* with DMI and milk fat content (92, 96). One unclassified genus belonging to *Clostridiales* showed a positive correlation with milk yield in the present study. Previous reports have shown substantial differences in abundance of

these taxa in beef steers with high and low residual feed intake (111).

Our study found that the tested mixed-phytogenic has the potential to stabilize rumen pH which may be beneficial especially for ruminants in intensive grain-based feeding systems. An increase in *Firmicutes-to-Bacteroidetes* ratio in response to mixed phytogenic substances reveals their synergistic potential to increase milk fat yield in buffaloes. The significant increase in omega-3 and numeric increase in PUFA in response to MP15 and MP25 may be beneficial from a human health perspective. Our study provides new information regarding the potential effect of a mixed phytogenic on the rumen microbial population, particularly rumen bacteria, and their potential association with fermentation and milk performance parameters. The use of tested mixture of different phytogenics could lead to improvements in production performance and digestive health of buffaloes. However, further studies on larger cohorts are required to solidify these first results and explore the shift in the rumen bacteriome and their impact on production related traits in depth.

CONCLUSIONS

Supplementation of MP increased rumen pH and n-3 fatty acid content of milk, while decreasing its stearic acid content. Additionally, MP promoted bacteria that are positively associated with milk and fat yield (*Firmicutes-to-Bacteroidetes ratio, Pseudobutyrivibrio, Butyrivibrio,* and *Succinivibrioanceae*). Overall, our findings provide new insight into the modulation of rumen bacteriome caused by a mixed phytogenic feed additive in water buffaloes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, no. PRJNA564158.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics committee of the Chinese Academy of Agriculture Sciences, Guangxi Buffalo Research Institute, China.

AUTHOR CONTRIBUTIONS

FH and CY: conceptualization. HE and ZT: data curation. FH, ZT, and HE: formal analysis. CY: funding acquisition, supervision, and validation. FH: investigation and writing–original draft. FH, HE, ML, LP, KP, and XL: methodology. XL and CY: project administration. ZT, ML, LP, KP, and CY: resources. FH and HE: software. FH, HE, and CY: writing–review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00569/full#supplementary-material

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Camelina sativa L. Oil Mitigates Enteric *in vitro* Methane Production, Modulates Ruminal Fermentation, and Ruminal Bacterial Diversity in Buffaloes

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This study was aimed to evaluate the effects of *Camelina sativa* oil (CO) on fermentation kinetics and methane (CH_4) production in rations with different roughage (R) to concentrate (C) ratios. Three total mixed rations (TMRs) were used as substrates (R70:C30, R50:C50, and R30:C70) supplemented with different levels of CO (0, 2, 4, 6, and 8% on dry matter basis) in an *in vitro* batch culture system. The enteric CH_4 production was determined at different times of incubation while fermentation parameters were measured at the end of incubation. Results revealed that CO significantly decreased ($P < 0.05$) CH_4 production at 48 h in medium (R50:C50) and low- (R30:C70) roughage diets than control. Camelina oil at all levels significantly ($P < 0.05$) affected ammonia nitrogen (NH_3-N) and microbial protein (MCP) in all rations. Propionate concentration was increased by supplementing 8% CO to R70:C30 TMR, but it decreased with increasing levels of CO for low- and medium-roughage diets. Acetate concentration was significantly ($P < 0.05$) higher at 4% CO supplementation, but it decreased with 8% CO level in R30:C70 TMR. For all rations, CO decreased ($P < 0.001$) total bacteria, protozoa, and methanogens. Total fungi counts were affected by CO in all rations, especially with a 6% level in two rations (R30:C70 and R50:C50) and 8% level with high-roughage ration (R70:C30). Supplementation of CO in medium-roughage ration (R50:C50) showed a linear ($P < 0.05$) decrease in bacterial richness and evenness indices along with Shannon diversity as compared to the control. Moreover, CO also increased *Firmicutes* to *Bacteroidetes* ratio in all TMRs more effectively at higher levels. Camelina oil also affected the relative abundance of *Prevotella* in both low- and medium-roughage diets while increasing the abundance of *Ruminobacter* and *Pseudobutyrivibrio*. The present study concluded that CO enhanced fermentation kinetics while decreasing enteric *in vitro* CH_4 production from fibrous diets. Thus, it may be considered as a potentially effective and environmentally friendly way of mitigating CH_4 emission from livestock.

Keywords: camelina oil, methane, rumen fermentation, methanogenesis, bacterial diversity

INTRODUCTION

Rumen fermentation is a major contributor to enteric methane (CH_4) in global greenhouse gas (GHG) emissions and considered an important player in the climate-change scenario. Methane mitigation has been keenly sought as a potential strategy to reduce GHG emissions while improving overall feed efficiency in ruminants. It is mainly targeted to mediate rumen biohydrogenation by diverting metabolic hydrogen away from methanogenesis toward the production of volatile fatty acids (VFA) that can potentially enhance production efficiency in ruminants and reduce environmental hazards. An earlier study has proposed that limiting methanogenesis can subsequently increase the production of microbial biomass (1). Moreover, excess metabolic hydrogen escaped from methanogenesis can be incorporated into NADH, which could be subsequently utilized in VFA synthesis and other fermentation end products in the rumen (2, 3).

Various methane mitigation strategies have been employed to control CH_4 formation in the rumen including use of chemicals (4), plant extracts (5, 6), and supplementation of vegetable oils (7–9). Compared with other methane mitigation strategies, supplementation of dietary fat or vegetable oils is potentially advantageous in terms of increasing dietary energy in high-producing animals while limiting CH_4 production (10, 11). Recently, the trend of incorporating fats as a source of dietary energy in place of carbohydrates is becoming popular, which also contributes to CH_4 mitigation (12). The unsaturated fatty acid contents of plant seeds and/or their oils can desirably influence the biohydrogenation process in the rumen (13).

The effect of plant oils such as rapeseed, sunflower, soybean, and linseed oils on rumen fermentation in diets with different roughage: concentrate ratios (R:C ratio) has been extensively studied (14–17). Studies have revealed that oilseeds can be one of the efficient ways to reduce enteric CH_4 production from ruminants as they can mitigate CH_4 by directly inhibiting rumen protozoa and methanogens while increasing biohydrogenation of polyunsaturated fatty acids (PUFA) to act as a sink for hydrogen produced by rumen microbes (8, 16, 18). The oil of *Camelina sativa* L. seed (CS), also known as false flax seed, is a rich source of unsaturated fatty acids (68–74%) especially oleic, linoleic, and linolenic acids (19, 20). This rich unsaturated fatty acid profile makes CS a high-quality fat supplement for ruminants. Moreover, CO is a quite stable oil despite having high PUFA contents owing to its rich antioxidant profile (21). Several studies have reported the use of CS meal or cake as a protein source in beef, lactating sheep, and dairy cow rations (22–24) while few have also used whole seed as a feed supplement (14, 20).

Studies have reported the nutritional benefits of CS on animal performance, but information regarding its potential effects on fermentation kinetics and ruminal microbiota is limited (13). Few studies have investigated the effect of oil extracted from whole camelina seed in dairy cows fed grass silage (25) and lambs (15). No study is available on the effect of CO on methane mitigation in high fibrous diets.

We hypothesized that PUFA contents (linolenic acid) of CO could potentially reduce CH_4 production by mediating rumen biohydrogenation and microflora. Therefore, we aimed to evaluate the effect of camelina oil on *in vitro* CH_4 production, rumen fermentation, and microbial populations in total mix rations (TMRs) with different roughage-to-concentrate ratios.

TABLE 1 | Ingredients and chemical composition (g/kg DM) of the three total mixed rations with different elephant (R) to concentrate (C) ratios ($N = 3$).

Items	Ingredient		Ration (R:C)		
	Elephant grass	CFM[a]	70:30	50:50	30:70
Ingredient					
Elephant grass			700	500	300
Crushed corn			180	300	420
Wheat bran			33	55	77
Soybean meal			75	125	175
Shell powder			1.5	2.5	3.5
Dicalcium phosphate			4.5	7.5	10.5
Sodium chloride			3	5	7
Minerals/vitamins mixture[b]			3	5	7
Chemical composition					
Dry matter	966	947	960	956	952
Organic matter	872	789	847	830	814
Crude protein	63	163	93	113	133
Neutral detergent fiber	879	595	794	737	680
Acid detergent fiber	532	126	410	329	248
GE[c] (kcal/kg DM)	4.16	3.95	4.06	4.01	4.06

[a]*CFM, Concentrate feed mixture.* [b]*Contained per kg: vitamin A 550 000 IU, vitamin E 3000 IU, vitamin D3 150 000IU, 4.0 g Fe (as ferrous sulfate), 1.3 g Cu (as copper sulfate), 3.0 g Mn (as manganese sulfate), 6.0 g Zn (as zinc sulfate), 80 mg Co (as cobalt sulfate).* [c]*GE, gross energy.*

MATERIALS AND METHODS

Substrates and Experimental Design

Three total mixed rations were prepared with different roughage-to-concentrate ratios, viz., 70:30, 50:50, and 30:70 coded as R70:C30, R50:C50, and R30:C70, respectively. Details of experimental TMRs are given in **Table 1**.

Camelina oil was obtained from Mountain Rose Herbs Company, Denmark. An emulsion (oil-in-water) of CO was prepared using the ultrasonic bath (Sonics Vibra-Cell™, USA) by suspending in distilled water (1:9; v/v). This emulsion was supplemented in substrates at four levels including 2% (125 μL), 4% (250 μL), 6% (375 μL), and 8% (500 μL) on dry matter (DM) basis. Two *in vitro* batch culture runs were carried out separately for each ration with oil supplement. Each oil level was tested in three replicates with three blank vessels (no substrate) for each run (in total 6 replicates). Five-hundred milligram of each ration was taken in the bottle (180 mL) as a substrate, and an artificial buffer solution was added, followed by mixing ruminal fluid at a 4:1 ratio as reported previously (26). The buffer solution was prepared 1 day before starting the fermentation. The rumen fluid collected from three cannulated water buffaloes (Murrah × Chinese local with an average body weight of 450 kg) fed the same roughage and concentrate diet was used for *in vitro*

fermentation. The ruminal digesta was collected from different places of the rumen in clean containers and strained through four layers of cheesecloth to get rumen fluid while flushing with CO_2. A 50-mL ruminal buffer solution was injected into each bottle with flushing CO_2, and after sealing, the bottles were kept in a pre-warmed incubator (at 39°C) for the next 48 h.

Sample Collection

During the incubation period, a 10-μL gas sample was taken from each bottle with a gas-tight syringe and manually injected into the GC system to determine the CH_4 concentration at 3, 6, 9, 12, 24, and 48 h of incubation using the GC system (7890A, Agilent Technologies, USA) with a capillary column measuring 30 m × 0.32 × 0.25 mm film thickness (GC-14B, Shimadzu, USA). Five different concentrations of pure CH_4 gas standards were used to develop calibration curves for the calculation of total CH_4 produced. The total gas production volume was measured by a glass syringe (100 mL) before each CH_4 measurement (data of gas production not shown).

After 48 h of incubation, the pH of rumen fluid was measured by a pH meter (HI 9024C; HANNA Instruments, Woonsocket, Rhode Island, USA), immediately after the opening of bottles. Samples of rumen fluid were separated into labeled plastic tubes (15 mL) for the analyses of microbial crude protein (MCP), ammonia nitrogen (NH_3-N), and volatile fatty acid fractions including acetic (C2), propionic (C3), butyric (C4), and valeric (C5) acids and their isomers including isobutyric (iC4) and isovaleric (iC5) acids. After that, all the samples were stored at −20°C until further processing.

Chemical Analyses

The fatty acid profile of camelina seed oil samples was analyzed as described previously (27) and presented in **Table 2**. Briefly, 1.0 mL of n-hexane was added to 15 mg of CO and vortexed for 30 s followed by the addition of 1 mL of sodium methoxide (0.4 mol). The mixture was allowed to settle for 15 min after vortexing for 30 s. The upper phase, containing the fatty acid methyl ester (FAME), was recovered and analyzed by an Agilent 7890B gas chromatography (GC-FID) with a polar capillary column SP®-2560 100 m, 0.25 mm id, 0.2 μm film thickness. Helium was used as a carrier gas at a flow rate of 20 cm s-1 and split ratio 100:1. The column temperature profile was held at 100°C for 5 min, ramp to 240°C at 4°C min-1, and held at 240°C for 30 min. A sample volume of 1.0 μL was injected. The FAME was identified by comparing their relative and absolute retention times with FAME standards (from C4:0 to C24:0).

The proximate analysis of TMRs was carried out for DM (ID: 934.01), ash (ID: 942.05), and nitrogen (ID: 954.01), and ether extract (ID: 920.39) according to AOAC procedures (28). The TMR samples were also analyzed for neutral detergent fiber (NDF) and acid detergent fiber (ADF) (ID: 973.18), as described by Van Soest et al. (29) using an ANKOM^{2000} Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA). Neutral detergent fiber content was analyzed with heat-stable α-amylase and sodium sulfite per sample in the neutral detergent solution. The NDF and ADF were expressed, inclusive of residual ash.

TABLE 2 | Fatty acid profile of oil isolated from *camelina sativa* (L) seeds (mean ± SD) (*N* = 3).

Fatty acid	Concentration (g/kg[a])
Palmitotate acid	70.9 ± 3.49
Stearic acid	28.1 ± 7.28
Oleic acid	168.7 ± 9.54
Linoleic acid	178.1 ± 11.6
Linolenic acid	302.5 ± 1.45
Arachidic acid	26.1 ± 2.02
Arachidonic acid	139.5 ± 5.23
Eicosadienoic acid	25.4 ± 3.62
Eicosatrienoic acid	16.8 ± 0.29
Behenic acid	6.2 ± 2.14
Saturated fatty acids	131.3 ± 14.93
Unsaturated fatty acids	831.0 ± 31.73
Other	37.7 ± 5.62

[a]*Concentration based on the total areas of the identified peak.*

The gross energy (GE) of rations was determined by a bomb calorimeter (PARR Calorimeter, USA).

Samples of VFA fractions in rumen fluid (C2, C3, C4, C5, iC4, and iC5) were measured using the GC system (Model 7890A, Agilent Technologies, USA; column temperature = 120°C, injector temperature = 180°C, detector temperature = 180°C). 0.5 mL of meta-phosphoric acid (25%) was added to 1 mL of filtrate then centrifuged (1,2000 × g for 10 min), and supernatant (920 μL) plus 80 μL crotonic acid (as internal standard) in a GC bottle for a volatile fatty acid determination as reported previously (30). A portion of 4 mL filtrate was acidified with 4 mL of HCl (0.2 mol/L) and stored in a freezer (−20°C) for NH_3-N using the indophenol method (31). For the determination of MCP, a 5-mL filtrate was then centrifuged at 800–1,000 rpm for 5 min at 4°C to remove feed particles. Then, 1.5 ml of supernatant was centrifuged at 12,000 rpm (4°C) for 15 min to collect the microbial biomass. After that, 0.5 mL (0.25 N) NaOH was added to the microbial biomass, mixed, and heated at 100°C in a water bath for 20 min. After this treatment, the mixture was centrifuged at 12,000 rpm (4°C) for 30 min, and the supernatant was collected for microbial CP analysis using the colorimetric method. Briefly, 100 μL supernatant was added to 5 mL Coomassie Brilliant Blue (G250, 95% ethanol, 85% phosphoric acid, and double-distilled water) and mixed well. Absorbance at 595 nm was checked by a 721 spectrophotometer colorimeter using 1 mg/ml bovine serum albumin solution (Sigma-Aldrich Co., LLC, St. Louis, Missouri, USA) as a standard equivalent (32). DNA of ruminal microbes was extracted from 1 mL of frozen samples of batch culture filtrate using the CTAB bead beating method (33).

Determination of Microbial Populations Using Real-Time PCR

Microbial populations in batch culture filtrate were determined through quantitative real-time PCR (qRT-PCR) by using a Roche LightCycler 480 RT-PCR machine (Roche, Basel, Switzerland).

TABLE 3 | PCR primers for real-time PCR assay.

Target strain	[a]Pre/post primer	Primer sequence	Amplification length (bp)	References
Total bacteria	F	CGGCAACGACCGCAACCC	130	(35)
	R	CCATTGTAGCACGTGTGTAGCC		
Total fungi	F	GAGGAAGTAAAAGTCGTAACAAGGTTTC	120	(35)
	R	CAAATTCACAAAGGGTAGGATGATT		
Total protozoa	F	GCTTTCGWTGGTAGTGTATT	223	(34)
	R	CTTGCCCTCYAATCGTWCT		
Total methanogens	F	TTCGGTGGATCDCARAGRGC	140	(36)
	R	GBARGTCGWAWCCGTAGAATCC		

[a]*F, forward; R, reverse.*

For the determination of methanogens and bacteria, we used a previously reported 16S-rRNA primer while for anaerobic fungi and protozoa, 18S-rRNA primers were used (34–36). Details of primers used in our study are presented in **Table 3**, while remaining procedures, including RT-PCR amplification profile and reaction mixture, were performed as reported in our previous study (8).

Sequencing of 16S-rRNA Gene for Determination of Rumen Bacterial Diversity

The DNA samples from one lower level (2%) and one higher level (8%) of CO were used for 16S rRNA gene sequencing to determine bacterial diversity. High-throughput (Illumina MiSeq) sequencing of the 16S rRNA gene was carried out using barcoded primers for the V3–V4 region (37). DNA libraries were sequenced using a 2 × 300 paired-end sequencing module (Illumina, San Diego). The taxonomic assignment of cleaned sequences was performed by aligning them against the SILVA database (Release128) using the Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/). Data about operational taxonomic units (OTU) were grouped taxonomically (at phylum and genus) for all treatment groups. The taxonomic classification of rumen bacteria in the steps mentioned above was performed as previously reported (38) using Qiime software (http://qiime.org/scripts/assign_taxonomy.htmL). Rarefaction curves and community bar plots were generated using R software (v2.3.2). Moreover, MOTHUR (V 1.31.2) software was used to analyze alpha diversity parameters.

Statistical Analysis

For each TMR, values recorded from three replicates of incubation run were averaged. Thus, within each TMR, there were six replicates per oil level (each corresponding to the average value recorded at two incubation runs), and each replicate was considered as an experimental unit. The effect of treatment on *in vitro* CH_4, rumen fermentation, and alpha bacterial diversity parameters of each TMR was analyzed through the PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA, version 14, 2015) using the following mixed model:

$$\text{Yijk} = \mu + \text{TMRi} + \text{COj} + \text{Eijk} \quad (1)$$

where Yijk = is every observation of the *i*th TMR type (TMRi) with *j*th CO level (COj); μ is the overall mean; Eijk is the experimental error. Copy numbers of protozoa, methanogens, bacteria, and fungi were log-transformed, and then a Poisson regression model was fitted using the PROC GENMOD of SAS. The level of statistical significance for all analyses was $P < 0.05$.

RESULTS

Chemical Composition of Diets and Fatty Acid Profile of Camelina Oil

Increasing the level of roughage ratio (elephant grass) in TMR decreased crude protein and increased NDF and ADF contents (**Table 1**). However, DM, OM, and GE were similar among the three TMRs. Analysis of fatty acid profile revealed linolenic acid as the most abundant fatty acid followed by linoleic, oleic, and arachidonic acids in CO (**Table 2**). Total unsaturated fatty acid (UFA) concentrations of CO were far greater than the total saturated fatty acids (831 vs. 131 mg/kg).

In vitro Methane Production

Treatment significantly affected the *in vitro* CH_4 production in three rations at different hours of incubation (**Table 4**). For ration R70:C30, CO linearly increased ($P < 0.05$) CH_4 production at 3, 6, 9, and 12 h of incubation than control. However, after 48 h of incubation CH_4 production was linearly ($P > 0.05$) decreased with supplementation of CO (**Table 4**). For ration R50:C50, CO almost linearly ($P < 0.001$) decreased CH_4 production after 24 h of incubation than control. For ration R30:C70, CO linearly increased ($P < 0.05$) CH_4 production at 3, 6, 9, and 12 h of incubation but linearly ($P < 0.001$) decreased CH_4 production after 48 h of incubation than control. Moreover, all TMRs exhibited a decrease in CH_4 production with all CO levels compared to the control after 48 h.

In vitro Rumen Fermentation Kinetics

Ruminal pH did not differ among treatments with both high- and medium-roughage rations, but increasing levels of CO linearly ($P < 0.0001$) decreased the pH in low-roughage ration (**Table 5**). Dietary CO levels, as well as the type of ration, significantly affected NH_3-N and MCP concentrations (**Table 5**). The concentrations of NH_3-N and MCP were increased with the

TABLE 4 | *In vitro* methane (CH_4) production (mL) kinetics of three total mixed rations supplemented with different levels of camelina oil ($N = 6$).

Time/h	Oil levels					SEM	*P*-value
	0%	2%	4%	6%	8%		
Roughage:concentrate (70:30)							
3	9.73b	9.99b	10.38a	9.91b	10.46a	0.075	0.002
6	10.53c	11.25ab	11.15b	11.22ab	11.64a	0.094	0.001
9	12.39c	13.71ab	14.23a	13.45b	13.80ab	0.155	0.001
12	14.78b	15.14ab	15.40a	15.30ab	15.35a	0.084	0.130
24	21.23	22.16	22.34	21.95	22.33	0.187	0.312
48	28.72	27.31	28.41	27.70	26.74	0.406	0.566
Roughage:concentrate (50:50)							
3	9.39	9.76	9.59	9.78	9.77	0.056	0.116
6	11.60	11.44	11.61	11.92	11.78	0.075	0.312
9	13.35	13.98	14.15	14.48	14.46	0.103	0.196
12	16.99	16.61	17.17	16.68	16.85	0.193	0.905
24	24.04a	23.09b	22.76b	23.16b	22.52b	0.137	0.001
48	26.45a	25.60b	25.48bc	25.63b	24.86c	0.130	0.001
Roughage:concentrate (30:70)							
3	9.21c	9.51b	9.47bc	9.44bc	9.81a	0.053	0.003
6	10.68c	10.92bc	10.98abc	11.23ab	11.25a	0.059	0.005
9	12.93b	13.13b	13.40ab	13.84a	13.47ab	0.097	0.024
12	15.43c	15.66bc	15.74bc	15.52a	16.17ab	0.111	0.007
24	24.78	24.66	24.54	24.91	24.53	0.127	0.876
48	30.78a	29.89ab	30.30ab	29.59b	29.18b	0.198	0.058

Arithmetic mean in the same row within each ration with different letters differ significantly ($P < 0.05$).

supplementation of CO in low- and medium-roughage rations. On the other hand, both NH_3-N and MCP concentrations were linearly decreased with CO supplementation in the high-roughage ration. The concentration of total VFAs increased with the increase in concentrate ratio in TMRs. Dietary supplementation of CO at 4% in high-roughage ration showed a significant increase in total VFAs. For ration R70:C30, propionic acid was linearly increased ($P < 0.016$) with an increasing level of CO; however, the A/P ratio was decreased ($P < 0.05$) with 8% CO as compared to the control. For the R50:C50 ration, 2% CO increased ($p < 0.018$) the concentration of C3, while higher levels showed a negative effect on C3 yield. The C4 ($P < 0.003$), C5 branched-chain VFA ($P < 0.031$), and C5 linearly ($P < 0.001$) decreased with the increasing level of CO. For R30:C70 TMR, 4% of CO significantly increased the cross C2, C4, and C5 branched-chain VFA and C5, while significantly decreasing ($P < 0.014$) the C3 concentration.

In vitro Rumen Microbial Populations

According to qRT-PCR results, type of ration and the level of CO significantly (linear and quadratic effects, $P < 0.05$) affected rumen microbial populations (**Table 6**). The number of protozoa, methanogens, and total bacteria was linearly and quadratically decreased ($P < 0.0001$) with CO supplementation in all rations. However, 8% CO increased the number of protozoa, methanogens, and total bacteria as compared to the control. Moreover, CO affected the total fungal counts in all rations, especially at 6% in the low-roughage ration (R30:C70) and 8% level in the high-roughage ration (R70:C30).

In vitro Bacterial Diversity

Taxonomy Statistics and Rarefaction Curves

A total of 3164 OTU were identified through analysis of 16S-rRNA gene sequence data in the three TMRs supplemented with three CO levels (0, 2%, and 8%). The taxonomic data revealed 23 phyla, 40 classes, 86 orders, 157 families, 364 genera, and 719 species of rumen bacteria detected in rumen filtrate. Shared and unique OTU for three rations with different levels of CO are presented in **Figure 1**. The total number of OTU increased in the high-roughage ration (R70) in both control and supplemented groups. The higher number of unique OTUs individually identified in the control group was observed with a medium-roughage ration followed by low CO level with high- and low-roughage rations, respectively. The number of observed species (sobs) at the OTU level was greater in the high-roughage ration (R70:C30) as compared to other groups (**Figure 2**).

Bacterial Diversity Indices

Results of alpha bacterial diversity indices are presented in **Table 7**. For the R70:C30 diet, the richness (sobs, ace, and Chao) and diversity (Shannon and Simpson) indices were increased ($P > 0.05$) with supplementation of the lower level of CO (2%) as compared to the high level (8%) and control in a high-roughage diet (R70). On the other hand, the treatment showed a linear ($P < 0.05$) decrease in all richness and evenness indices as well

TABLE 5 | *In vitro* fermentation parameters of three total mixed rations supplemented with different levels of camelina oil ($N = 6$).

Items*	Oil levels					SEM	*P*-value
	0%	2%	4%	6%	8%		
Roughage: concentrate (70:30)							
pH	6.75	6.75	6.74	6.70	6.74	0.010	0.644
NH_3-N	7.93a	7.92a	7.80a	6.25b	6.69ab	0.227	0.031
MCP	12.08a	11.89a	10.87ab	10.87ab	9.24b	0.351	0.067
TVFA	24.24	22.22	23.87	25.19	24.90	0.467	0.299
C2	11.16	10.11	10.87	11.53	11.29	0.221	0.312
C3	7.94ab	7.40b	7.89ab	8.43ab	8.48a	0.155	0.016
isoC4	0.47	0.44	0.47	0.47	0.46	0.007	0.609
C4	3.99	3.66	3.96	4.11	4.02	0.079	0.462
isoC5	0.41	0.37	0.42	0.40	0.40	0.011	0.705
C5	0.26	0.22	0.25	0.25	0.26	0.006	0.596
A/P	1.40a	1.36ab	1.37ab	1.37ab	1.33b	0.008	0.050
Roughage: concentrate (50:50)							
pH	6.75	6.76	6.75	6.76	6.74	0.003	0.134
NH_3-N	8.84a	7.77b	7.43b	8.92a	9.29a	0.202	0.005
MCP	9.20ab	8.57b	8.70b	9.57ab	10.79a	0.290	0.099
TVFA	26.23	26.27	25.32	26.02	25.59	0.157	0.230
C2	11.66	11.49	11.17	11.42	11.19	0.078	0.229
C3	8.67ab	8.96a	8.32b	8.63ab	8.58ab	0.082	0.018
isoC4	0.50a	0.49ab	0.47c	0.47bc	0.46c	0.004	0.003
C4	4.51	4.48	4.59	4.71	4.60	0.042	0.478
isoC5	0.51a	0.49ab	0.45b	0.46b	0.45b	0.008	0.031
C5	0.37a	0.35ab	0.32c	0.34bc	0.31c	0.006	0.001
A/P	1.35	1.28	1.35	1.33	1.31	0.011	0.293
Roughage: concentrate (30:70)							
pH	6.68ab	6.70a	6.66bc	6.64d	6.62dc	0.007	<0.0001
NH_3-N	9.16a	6.31b	9.48a	9.33a	11.07a	0.414	0.0021
MCP	10.18	9.89	9.15	10.33	10.12	0.257	0.6479
TVFA	30.06a	28.95ab	30.24a	29.69a	27.88b	0.253	0.0099
C2	12.82a	12.40ab	12.91a	12.65a	11.87b	0.112	0.0132
C3	10.31a	9.75ab	10.21a	10.06a	9.32b	0.108	0.0144
isoC4	0.543a	0.543a	0.558a	0.533ab	0.507b	0.006	0.0504
C4	5.33	5.13	5.38	5.38	5.16	0.047	0.2749
isoC5	0.647ab	0.680a	0.687a	0.645ab	0.607b	0.011	0.1263
C5	0.420b	0.453ab	0.495a	0.427b	0.417b	0.010	0.0392
A/P	1.24	1.27	1.27	1.26	1.28	0.008	0.7449

*Arithmetic mean in the same column within each ration with different letters differ significantly ($P < 0.05$). *Items: NH_3-N, ammonia-N (mg /100 mL); MCP, microbial crud protein (mg/mL); VFA, volatile fatty acids (mmol/L); C2, acetic acid (mmol/L); C3, propionic acid (mmol/L); C4, butyric acid (mmol/L); C5, valeric acid (mmol/L); iC4, isobutyric acid (mmol/L); iC5, isovaleric acid (mmol/L); A/P, acetate to propionate ratio.*

as Shannon diversity in the medium roughage ration (R50:C50). However, the low-roughage ration (R30:C70) showed a similar trend for richness and Shannon indices that were observed in the high-roughage diet (R70) supplemented with a 2% CO level as compared to others. The ace index was significantly ($P < 0.042$) increased with a 2% CO level.

The Relative Abundance of Bacterial Phyla

The relative abundance of different bacterial phyla observed in our study is presented in **Figure 3**. Results revealed five major bacterial phyla, namely, *Bacteroidetes* (41.77–51.08%), *Firmicutes* (30.44–43.26%), *Proteobacteria* (4.10–8.95%), *Spirochaetes* (1.33–4.09%), and *Kiritimatiellaeota* (1.14–2.60%), which were observed in all three rations. Moreover, two unique phyla *Fibrobacteres* and *Lentisphaerae* were only detected in the R70:C30 diet. The phylum *Synergistetes* was observed in the medium-roughage (R50:C50) diet only. Three bacterial phyla (*Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) constituted >98% of total rumen bacteriome. The type of ration and level of CO significantly ($P < 0.05$) affected

TABLE 6 | Ruminal microbiota population (log colony forming units/mL) of three total mixed rations supplemented with different levels of camelina oil ($N = 3$).

Ration	Level	Protozoa	Methanogens	Bacteria	Fungi
R70:C30	0	8.21a	7.08a	20.37a	5.11ab
	2	7.49b	6.51b	20.24b	5.16a
	4	7.03c	6.67b	18.31e	5.17a
	6	7.47b	6.67b	19.81c	5.13ab
	8	6.79d	6.32c	19.05d	5.03b
	SEM	0.017	0.022	0.0004	0.044
	Oil linear	<0.0001	<0.0001	<0.0001	0.225
	Oil quadratic	<0.0001	0.001	<0.0001	0.032
R50:C50	0	8.63a	6.95a	21.15a	5.19
	2	8.50b	6.27d	19.81e	5.11
	4	8.16d	6.60c	20.18c	5.12
	6	8.31c	6.75b	20.74b	5.07
	8	7.89e	6.28d	20.08d	5.11
	SEM	0.009	0.017	0.0003	0.043
	Oil linear	<0.0001	<0.0001	<0.0001	0.132
	Oil quadratic	<0.0001	0.003	<0.0001	0.281
R30:C70	0	7.51b	6.54c	18.49b	5.18a
	2	7.45c	7.04a	17.95e	5.24a
	4	7.07d	5.98d	17.96d	5.20a
	6	7.02d	6.59c	17.98c	5.02b
	8	7.77a	6.73b	19.49a	5.13ab
	SEM	0.016	0.021	0.0001	0.043
	Oil linear	0.017	0.375	<0.0001	0.016
	Oil quadratic	<0.0001	<0.0001	<0.0001	0.865
***P*-value Ration**					
	SEM	0.005	0.001	0.0003	0.002
	Linear	0.0003	<0.0001	<0.0001	<0.0001
	Quadratic	<0.0001	<0.0001	<0.0001	<0.0001
Oil effect					
	SEM	0.006	0.001	0.0003	0.002
	Linear	<0.0001	<0.0001	<0.0001	<0.0001
	Quadratic	<0.0001	<0.0001	<0.0001	<0.0001

Arithmetic mean in the same column within each ration with different letters differ significantly ($P < 0.05$).

the bacterial populations. The population of *Firmicutes* ($P < 0.04$) and *Proteobacteria* ($P < 0.007$) was affected by the low and medium level of roughage in all treatment groups. However, supplementation of 2% CO increased ($P < 0.01$) the relative abundance of *Bacteroidetes* in the low-roughage ration. Moreover, the relative abundance of *Spirochaetes* was decreased with the increase in CO level in the high-roughage ration while no difference ($P > 0.18$) was observed in the low- and medium-roughage rations at all CO levels.

The Relative Abundance of Bacteria at the Genus Level

The effects of ration and CO on the relative abundance of bacterial genera are presented in **Figure 4**. We detected a total of 364 bacterial genera among which 11 major genera included *Rikenellaceae_RC9_gut_group* (9.5–15.25%), *norank_f_F08*, *Prevotella_1* (6.82–18.18%), *Ruminobacter* (2.32–6.59%), *unclassified_o_Clostridales* (2.91–5.40%), *Christensenellaceae_R-7_group* (2.41–4.34%), *Ruminococcaceae_NK4A214_group* (1.68–3.93%), *Pseudobutyrivibrio* (1.21–3.06%), *Ruminococcaceae_UCG-010* (1.71–2.76%), *norank_o_WCHB1-41* (1.14–2.57%), and *Succiniclasticum* (1.10–2.75%). These bacterial phyla constituted about 55% of the total bacteriome. The higher level of CO decreased ($P > 0.109$) the abundance of *Rikenellaceae_RC9_gut_group* in low- (R30) and medium- (R50) roughage diets. However, norank_f_F08 was decreased ($P < 0.007$) in high (R70) roughage than other diets. The abundance of the *Prevotella_1* genus was affected ($P > 0.135$) by both the type of ration and treatment. However, a higher CO level increased ($P < 0.013$) the relative abundance of *Ruminobacter* in low- (R30) and medium- (R50) roughage diets than low CO and control groups, but this genus showed similar abundance in high-roughage (R70) groups. Moreover, the abundance of *unclassified_o_Clostridales* was linearly increased ($P = 0.106$) in all three TMRs with the increase in CO as compared to the control. The *Ruminococcus* as cellulolytic bacteria (*Ruminococcaceae_NK4A214_group*) showed variation among different rations, and the highest ($P < 0.007$) abundance was observed in treated medium- (R50) roughage ration. However, *Ruminococcaceae_UCG-005* (ranged from 0.50 to 2.50%) showed a shift in high-roughage rations compared to other rations ($P < 0.004$). A low level of CO favored *Succiniclasticum* in the low roughage ration but reduced ($P > 0.495$) its abundance in the high-roughage diet (R70). The *Pseudobutyrivibrio* increased ($P < 0.013$) in low- and medium-roughage rations supplemented with a high CO level (8%), but no difference in its abundance was observed in high-roughage rations (both treated and untreated). However, a high level of CO increased ($P = 0.055$) the relative abundance of *Butyrivibrio 2* (ranged from 0.80 to 2.03%) in all TMRs.

DISCUSSION

Our hypothesis that supplementation of camelina oil (rich in PUFA) could affect ruminal bacterial community composition leading to subsequent changes in ruminal fermentation and metabolite production was proved in findings observed in this study.

Fatty Acid Profile of Camelina Oil

The analysis of the fatty acid profile of CO showed that linolenic acid was dominant fatty acid (>30%), and total unsaturated FA represented >83% of the total fatty acid contents. Previously, total unsaturated fatty acid concentrations up to 86.3, 87.6, and 87.5% have been reported in camelina oil in different studies (25, 39, 40), respectively. However, some studies have also reported lower unsaturated fatty acid fractions (especially, oleic, linoleic, and linolenic acid) ranging from 68 to 74% of total fatty acids (19, 20). These variations in oil composition

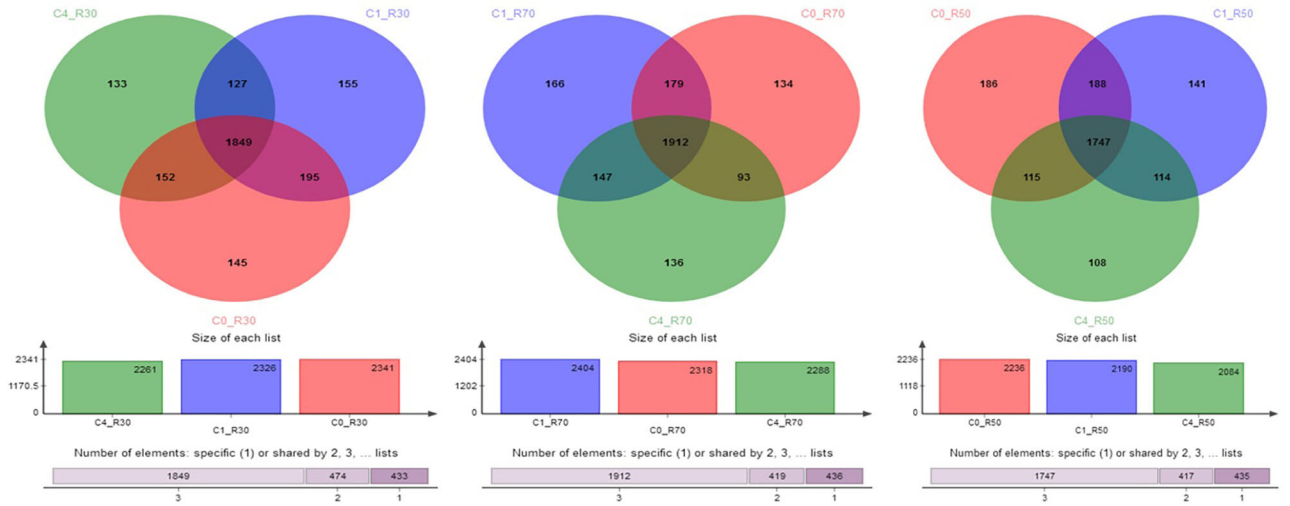

FIGURE 1 | Shared and unique OTUs across groups. C0_R70: control with 70% high roughage; C1_R70: 1% camelina oil level with 70% high roughage; C4_R70: 4% camelina oil level with 70% high roughage; C0_R30: control with 30% low roughage; C1_R30: M1: 1% camelina oil level with 30% low roughage; C4_R30: 4% camelina oil level with 30% low roughage; C0_R50: control with 50% medium roughage; C1_R50: M1: 1% camelina oil level with 50% medium roughage; C4_R50: 4% camelina oil level with 50% medium roughage.

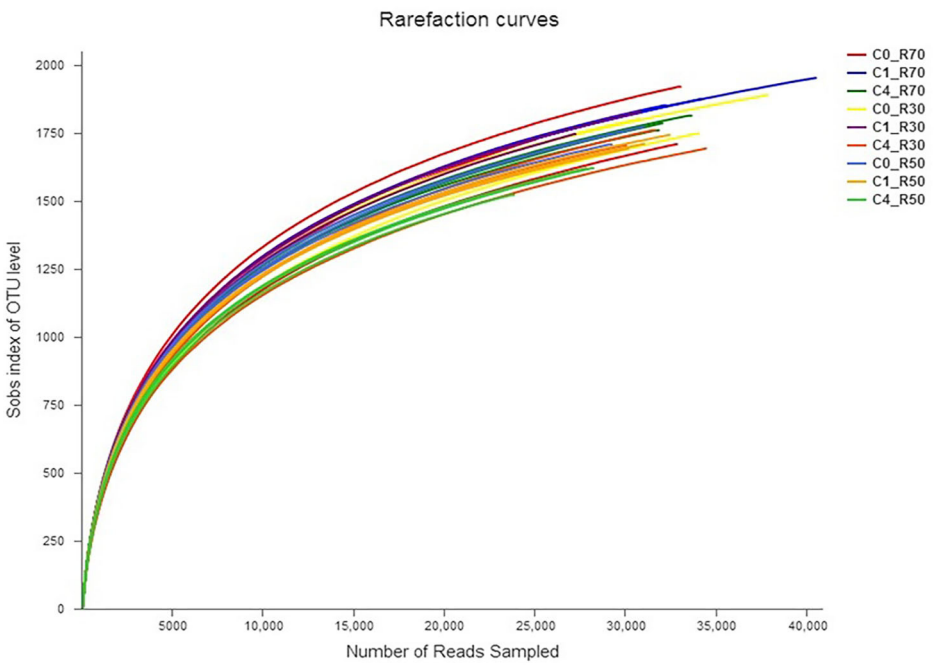

FIGURE 2 | Rarefaction curve showing bacterial richness across samples. C0_R70: control with 70% high roughage; C1_R70: 1% camelina oil level with 70% high roughage; C4_R70: 4% camelina oil level with 70% high roughage; C0_R30: control with 30% low roughage; C1_R30: M1: 1% camelina oil level with 30% low roughage; C4_R30: 4% camelina oil level with 30% low roughage; C0_R50: control with 50% medium roughage; C1_R50: M1: 1% camelina oil level with 50% medium roughage; C4_R50: 4% camelina oil level with 50% medium roughage.

are mainly due to different extraction methods and seed quality owing to different agro-climatic conditions of crop production. Our study observed a little bit lower levels of UFA (83%) in CO as compared to earlier report (87% UFA observed by (25). A high concentration of UFA can act as an alternate hydrogen sink during bio-hydrogenation (41) resulting in a decrease in CH_4 production in the rumen (42); however, it may also decrease fiber digestibility (42).

TABLE 7 | Effect of camelina oil supplementation and three total mixed rations alpha microbial diversity estimators ($N = 3$).

Level*	sobs	Shannon	Simpson	ace	Chao	coverage	Shannon eveness
Roughage: concentrate (70:30)							
C	1785.00	6.02	0.0076	2248.72	2292.80	0.983	0.804
L	1893.33	6.06	0.0078	2273.03	2312.65	0.986	0.804
H	1786.66	6.05	0.0078	2176.16	2219.93	0.986	0.808
SEM	43.80	0.056	0.0006	38.27	46.15	0.001	0.005
Linear	0.615	0.797	0.833	0.150	0.226	0.331	0.596
Quadratic	0.101	0.603	0.843	0.417	0.541	0.134	0.889
Roughage: concentrate (50:50)							
C	1748.00a	6.17a	0.005	2148.78a	2156.79	0.985a	0.826a
L	1715.33a	6.11a	0.005	2079.76ab	2104.00	0.986a	0.821a
H	1587.33b	6.01b	0.006	1980.15b	2002.11	0.983b	0.815b
SEM	23.79	0.017	0.0003	31.41	43.00	0.0004	0.002
Linear	0.002	0.001	0.034	0.009	0.042	0.022	0.010
Quadratic	0.813	0.536	0.493	0.527	0.805	0.023	0.362
Roughage: concentrate (30:70)							
C	1813.33	6.127	0.005	2205.30a	2214.71	0.987	0.816
L	1800.66	6.198	0.004	2238.12a	2276.18	0.984	0.826
H	1719.33	5.99	0.007	2110.98b	2154.61	0.986	0.804
SEM	31.01	0.052	0.001	27.91	41.67	0.001	0.006
Linear	0.060	0.056	0.039	0.025	0.190	0.920	0.091
Quadratic	0.793	0.168	0.193	0.164	0.199	0.026	0.134
***P*-value Ration**							
SEM	19.57	0.02	0.0003	18.94	25.20	0.0005	0.002
Linear	0.130	0.116	0.0003	0.091	0.109	0.930	0.017
Quadratic	0.0001	0.477	0.041	<0.0001	<0.0001	0.637	0.008
Camelina oil							
SEM	19.57	0.02	0.0003	18.94	25.20	0.0005	0.002
Linear	0.002	0.011	0.012	0.0002	0.006	0.882	0.077
Quadratic	0.108	0.214	0.668	0.333	0.311	0.440	0.393

Arithmetic mean in the same column within each ration with different letters differ significantly ($P < 0.05$).
**Level: C, control without CO; L, low, 2% CO; H, high, 8% CO level.*

Methane Production

In the present study, we evaluated the effect of increasing levels of CO on *in vitro* fermentation and methane production. Dietary lipids are one of the promising ways to mitigate methane production in ruminants (18). The use of lipids to reduce enteric CH_4 production is a better strategy as compared to chemical feed additives like monensin and antibiotics. Recently, a study has reported adverse effects of fatty acids, especially PUFA, on methanogenesis in the rumen (18). The anti-methanogenic effects of PUFA generally get intensified with the increase in double bond number per FA, as suggested by Czerkawski and Clapperton (43). These findings support our observation in the present *in vitro* study. Moreover, another study has shown more severe toxic effects of linolenic acid (C18:3) than linoleic acid (C18:3) contents of linseed on cellulolytic bacteria (44). Our findings on CH_4 production are consistent with this observation because linolenic acid contents were more than 30% of the total FA in CO used in our study. The decrease in CH_4 production was observed after 48 h of incubation in response to the supplementation of CO in all rations. Previously, Wang et al. (18) reported that camelina seeds decreased CH_4 (ml/g DM) in a ration having a roughage-to-concentrate ratio of 60:40.

Our findings are in agreement with Bayat et al. (17), who reported a decrease in daily CH_4 emission in lactating cows fed with different forage to concentrate ratios (low and high) supplemented with sunflower oil. Moreover, dietary supplementation of CO (at 10 g/kg DM) in a 50% roughage ration has shown to reduce CH_4 production up to 4.8%. Variable effects of unsaturated fatty acids on CH_4 emission might be associated with the nature of FA (double bond number per FA), type of oil (free oil or whole seed), and composition (roughage-to-concentrate ratio) of the rations (25).

Rumen Kinetics

It is the first report on the potential effects of CO on *in vitro* fermentation kinetics using a rumen inoculum from water buffalo. Therefore, due to the scarcity of available information on the subject matter, we compared our findings with studies that

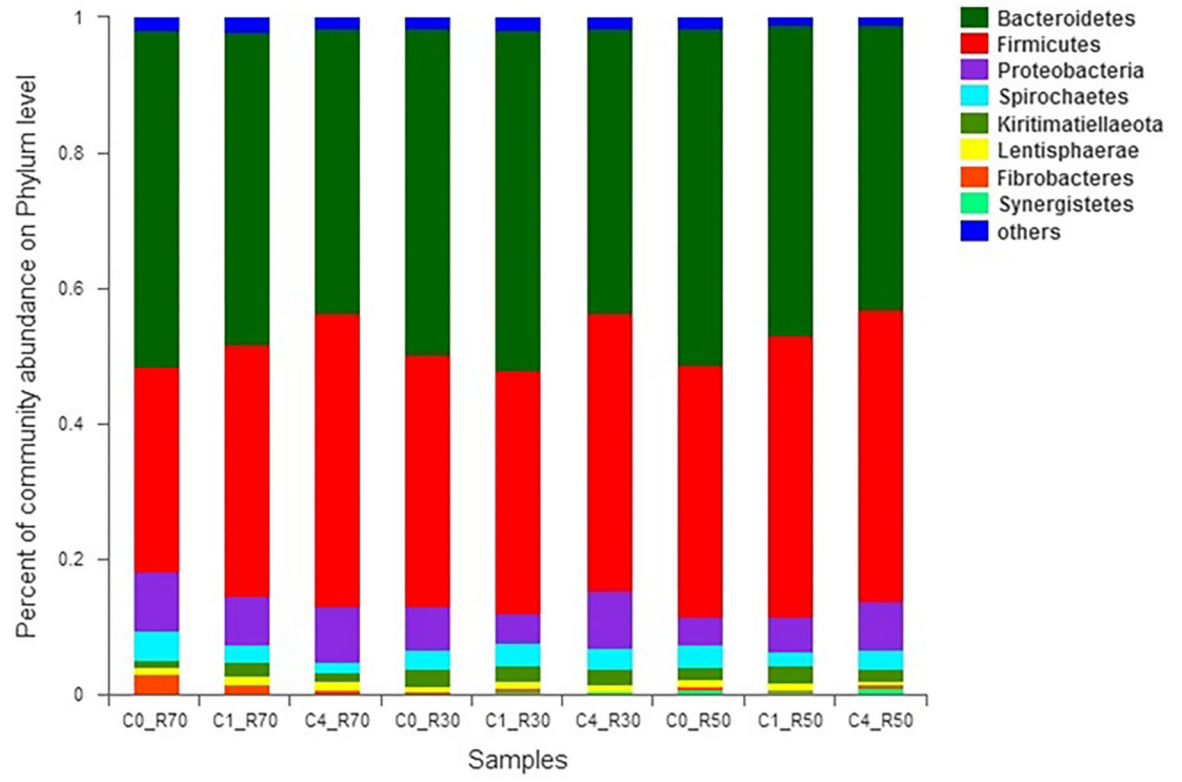

FIGURE 3 | Relative abundance of bacterial phyla across different treatment groups. C0_R70: control with 70% high roughage; C1_R70: 1% camelina oil level with 70% high roughage; C4_R70: 4% camelina oil level with 70% high roughage; C0_R30: control with 30% low roughage; C1_R30: M1: 1% camelina oil level with 30% low roughage; C4_R30: 4% camelina oil level with 30% low roughage; C0_R50: control with 50% medium roughage; C1_R50: M1: 1% camelina oil level with 50% medium roughage; C4_R50: 4% camelina oil level with 50% medium roughage.

used different parts of camelina seed (whole seeds or seed meal) or other vegetable oils. Rumen kinetics such as NH_3 and VFAs are major end products of rumen fermentation (45). As mentioned before, the majority of earlier studies have used camelina seeds (14, 20) or camelina meal or cake (23, 24), but only two reports are available regarding the use of camelina oil in dairy cattle (25) and fattening sheep (15). All levels of camelina oil linearly decreased rumen pH in R30:C70 ration in the present study; however, no effect of CO on pH was observed in other diets as reported previously (25). Moreover, NH_3-N was affected in all three diets and linearly decreased with a high concentrate ration. A similar pattern was observed regarding the concentration of MCP. However, Wang et al. (18) found that NH_3 was high after 24 h of incubation in 60% forage and 40% concentrate diet supplemented with camelina seeds. They suggested that the increase in NH_3 contents in rumen might indicate accelerated lysis of microbes or reduced nitrogen utilization. However, these findings are in disagreement with the present study, where NH_3 decreased in high-roughage diet, and this variation might be attributed to the diet composition (different R:C ratios), form (oil vs. seeds), and level of supplemental camelina. Another possible reason for the decrease in NH_3-N might be the inhibition of bacteria caused by antioxidant compounds of CO (46). Different essential oils and/or plant extracts have also shown to inhibit NH_3-producing bacteria (*Prevotella spp*. and *R. amylophilus*) up to 77% in sheep (47).

The VFAs are end-products of ruminal fermentation and provide ruminants with about 70% of metabolizable energy (48). The supplementation of CO increased propionate contents in R70:C30 while decreasing it in the other two diets except in the R50:C50 ration (only at 2% CO), which showed a higher propionate than the control group. Additionally, acetate production showed a similar trend like propionate, exhibiting the diet-specific effect of CO on total and individual VFA contents (49). Our findings are in agreement with previous study reporting an increase in the propionate and decrease in total VFA and acetate concentrations in a response to the supplementation of camelina seed (5%) in the diet (55% alfalfa hay: 45% concentrate) under *in vitro* conditions (50). In contrast, Hurtaud and Peyraud (20) observed a significant ($P < 0.001$) decrease in rumen acetate and increase ($P = 0.014$) in propionate in cows fed camelina seeds in the diet (59:41 R:C ratio). Our findings indicate that relatively higher amounts of fiber in the diet and level of CO supplementation can influence molar VFA proportions as well

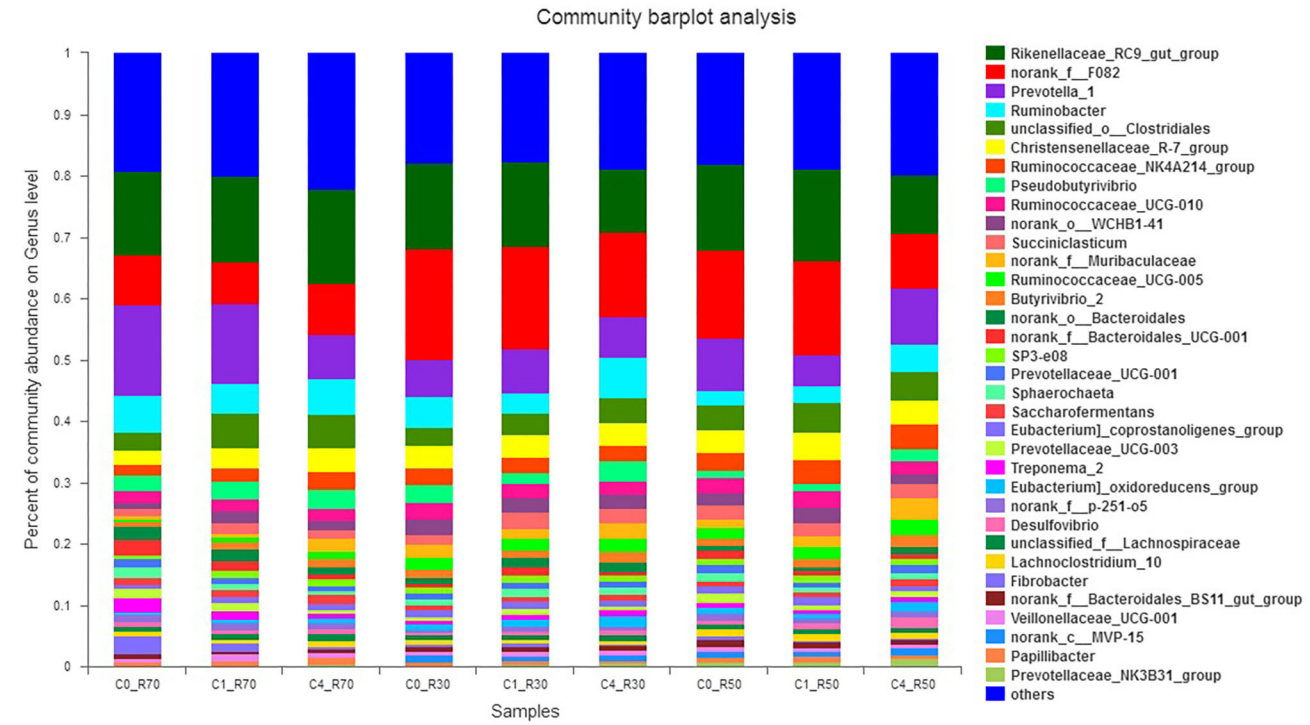

FIGURE 4 | Relative abundance of bacterial genera across different treatment groups. C0_R70: control with 70% high roughage; C1_R70: 1% camelina oil level with 70% high roughage; C4_R70: 4% camelina oil level with 70% high roughage; C0_R30: control with 30% low roughage; C1_R30: M1: 1% camelina oil level with 30% low roughage; C4_R30: 4% camelina oil level with 30% low roughage; C0_R50: control with 50% medium roughage; C1_R50: M1: 1% camelina oil level with 50% medium roughage; C4_R50: 4% camelina oil level with 50% medium roughage.

as fiber-degrading bacteria that are particularly sensitive to high-oil diets (44). Therefore, a decrease in acetate and an increase in MCP concentrations observed in R50:C50 and R30:C70 rations suggest a potential inhibition of fiber-degrading bacteria by CO (51).

Rumen Microbial Population

Our study revealed significant effects of supplementation of camelina oil on ruminal microbial populations, as shown in **Table 6**. Inhibition of specific microbial population observed in this study might be due to the direct toxic effects of CO on rumen microbes and/or higher non-fermentable fatty acids by replacing readily fermentable carbohydrates (52, 53).

The diet composition significantly affects the diversity and abundance of microbial populations owing to different nutrient contents and fermentation profiles (54). Similar findings have been reported earlier in cows fed high-roughage or high-concentrate diets (55). A significant decrease in methanogenic archaea was observed in goats when diet changed from alfalfa hay to a mixture of oats and alfalfa hay (56). In our study, camelina oil significantly affected the composition of ruminal microbiota, which is mainly attributed to the potential effects of its fatty acid contents. The significant decrease in the number of protozoa and methanogens well-correlated with the results of the CH_4 yield observed in this study. The rumen archaea use hydrogen to yield CH_4 to facilitate fiber digestion (57). The inhibition of methanogens is potentially beneficial owing to its significant association with methane emission and animal productivity (57).

The increasing levels of CO levels in diets had no effect on ruminal protozoa (58). However, the decrease in bacterial nitrogen and the number of cellulolytic bacteria have been observed in diets supplemented with 8% dietary lipids (50). Moreover, Bayat et al. (25) reported that CO exhibited no effect on protozoa, total bacteria, methanogens, fungi, and fiber-degrading bacteria. Variable results reported by different studies may be attributed to the dietary form of camelina used in the diet, composition of feed ingredients, roughage-to-concentrate ratio, and dose of oil supplements (25, 49, 59). Another explanation of our findings is the manipulation of rumen biohydrogenation as PUFA (such as linolenic acid) contents of CO could use hydrogen produced by rumen microbes serving as an alternate hydrogen sink (9).

Rumen Bacterial Diversity

Our study revealed significant effects of CO and ration (roughage-to-concentrate ratio) on ruminal bacterial diversity. Both dietary and treatment factors altered ruminal bacterial communities as well as their richness and diversity indices, as reported previously (13). In addition, shifts in ruminal microbial community and decrease in richness and diversity indices in response to supplementation of camelina seeds as compared to the dietary fat have been reported earlier (13). The study

suggested two possible reasons for these findings: (1) higher saturated fatty acid contents of dietary fat (Megalac) posed deleterious effects on rumen environment and (2) toxic effects of PUFA on rumen bacteria such as cellulolytic bacteria.

Our study revealed higher bacterial species richness both in high-roughage diets and in the lower level of CO as revealed by rarefaction curves. The four phyla, including *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Spirochaetes*, were detected as major phyla in our study, which is in agreement with earlier reports (13, 60). However, change in roughage-to-concentrate ratio favored two phyla *Fibrobacteres* and *Lentisphaerae* in the R70:C30 diet and one phylum *Synergistetes* in R50:C50 diet.

Two dominant phyla *Firmicutes* and *Bacteroidetes* constitute a major portion of rumen bacteriome. The *Firmicutes* produce propionate and butyrate, while *Bacteroidetes* produce short-chain fatty acids (SCFAs) by fermenting polysaccharides and cellulosic plant material with potential beneficial effects (61–63). In our study, an increase in *Firmicutes* to *Bacteroidetes* ratio was observed in all roughage rations more specifically at higher levels (8%) of CO. Earlier study has reported the potential association of this ratio of two major bacterial phyla (*Firmicutes*: *Bacteroidetes*) with energy homeostasis in animals (adipogenesis) and milk-fat yield in dairy cattle (64). An increase in this ratio in response to CO supplementation in our study correlated well with higher concentrations of propionate and butyrate observed in the present study. Moreover, acetate is produced by *Bacteroidetes* as a result of the fermentation of indigestible carbohydrates, which decreased in all treated TMRs. Camelina oil possesses abundant polyphenols and PUFA (mainly C18:3) contents that exhibited more obvious results against Gram -ve bacteria (13, 18, 44).

At the genus level, no data is available on the effects of the supplementation of CO on the rumen microbiome. Only one study has reported the effects of camelina seeds on microbial diversity (13). Our study revealed shifts in the relative abundance of the *Rikenellaceae RC9 gut group* with a high level of CO in low- and medium-roughage rations, which is in agreement with previous findings observed with the use of Tucumã oil in TMR (65). *Prevotella* was observed as a second major bacterial genus in our present study (**Figure 4**). Higher and lower levels of CO affected the relative abundance of *Prevotella* spp. that fluctuated among three rations. A low level of CO in the high-roughage ration (R70) showed a higher abundance of *Prevotella* spp. as compared to low- (R30:C70) and medium-roughage (R50:C50) rations. Contrarily, an increase in the relative abundance of *Prevotella* species (*P. bryantii* and *P. ruminicola*) in response to supplementation of higher levels of plant essential oils has been reported earlier (66). Moreover, dietary protein contents have shown to positively affect the relative abundance of *Prevotella* (60). However, the *Prevotella* abundance was decreased by 8% dietary ether extract, which is in agreement with our findings observed with a high level of CO in the present study (13). Variable findings observed in previous reports might be due to the type of oil, its levels, and the composition of diets. Moreover, studies have also revealed the supportive role of *Prevotella* in fiber digestion and utilization through facilitating other rumen microbes (13, 60, 66, 67). In our study, a decrease in acetate production with a high-roughage ration was observed in response to the shifting of *Ruminococcaceae_UCG-005* in high-roughage rations (60) possibly due to the toxic effects of linolenic acid contents (of camelina oil) on *Ruminococci* spp. (13).

Ruminal fermentation of starch by *Ruminobacter* and *Succiniclasticum* usually yields acetic and succinic acids (68). Our findings regarding the decrease of *Succiniclasticum* in the high-roughage ration are consistent with earlier report (60). On the other side, *Ruminobacter* abundance was decreased by a lower level of CO (2%) in low- and medium-roughage rations but increased with a high level of CO (8%) in all rations. Fermentation of polysaccharides by rumen bacteria (*Butyrivibrio* and *Pseudobutyrivibrio*) produces formic, butyric, and acetate acids in the rumen (69). The present study showed a higher level of butyric acid with supplementation of CO at 4 and 6% levels in all rations, which is mainly attributed to the increased number of *Butyrivibrio* species. Additionally, a decrease in butyric acid contents with the increase in roughage level was observed; however, the relative abundance of *Pseudobutyrivibrio* and *Butyrivibrio* showed a similar trend in all three TMRs and decreased with supplementation of CO at a lower level for low- and medium-roughage diets. However, Dai et al. (13) reported that supplementation of camelina seeds decreased ($P < 0.05$) *Butyrivibrio* without affecting the relative abundance of *Pseudobutyrivibrio*. A possible reason for decreased *Butyrivibrio* and *Pseudobutyrivibrio* with the low CO level and their increase with the high CO level might be attributed to the first shock of the toxic effects of linolenic acid on cellulolytic bacteria. Moreover, *Pseudobutyrivibrio* is more resistant than *Butyrivibrio* in the presence of C18:2n6, and C18:3n3, as well as *Butyrivibrio*, has significant negative association and sensitivity to toxic effects of PUFA (47, 70). However, previous study has reported a linear decrease in *Butyrivibrio* and *Pseudobutyrivibrio* with increasing levels of starch while the linear increase was observed with increasing fiber contents in the diet, which is consistent with our findings (60). This shows that *Butyrivibrio* and *Pseudobutyrivibrio* preferably ferment structural carbohydrates to yield energy. Additionally, similar functions of *Eubacterium* and *Butyrivibrio* regarding the fermentation of structural carbohydrates have been reported earlier (71). Conclusively, our study revealed desirable effects of camelina oil on rumen microbiota and fermentation kinetics while reducing CH_4 emission which is advantageous in terms of increasing feed efficiency and reducing environmental hazards.

CONCLUSIONS

Camelina oil reduced CH_4 production in total mixed rations, revealing its potential as a feed additive to mitigate overall methane emissions from livestock production. Supplementation of CO at the 8% level had adversely affected the diversity and evenness of bacterial populations. However, CO also increased *Firmicutes* to *Bacteroidetes* ratio in all rations particularly at a higher level of supplementation (8%). Camelina oil also negatively affected the *Prevotella* in both low- and medium-roughage diets while increasing

the abundance of *Ruminobacter* and *Pseudobutyrivibrio*, revealing its potential to favor the production of formate, butyrate, and acetate. Conclusively, our study revealed that supplementation of 4–6% CO in dairy rations is suitable to improve nutrient digestion and utilization while reducing CH_4 production. However, further *in vivo* studies are required to confirm our findings and to optimize the best level of CO to improve feed efficiency in ruminants while mitigating methane emanation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SRR10072455.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Guangxi Buffalo Research Institute, Chinese Academy of Agriculture Sciences, China.

AUTHOR CONTRIBUTIONS

HE: conceptualization, data curation, and writing—original draft. HE, ML, and FH: methodology. FH: software. CY: validation and supervision. HE and FH: formal analysis and investigation. ML, LP, XL, KP, and CY: resources. FH and CY: writing—review & editing. HE and ML: project administration. CY: funding acquisition. All authors contributed to the article and approved the submitted version.

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Mulberry Leaf Flavonoids Improve Milk Production, Antioxidant, and Metabolic Status of Water Buffaloes

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This study was aimed to evaluate the effect of mulberry leaf flavonoids (MLF) on oxidative stress, metabolic hormones, and milk production in Murrah buffaloes. Forty multiparous Murrah buffaloes (4 ± 1 lactations) with similar body weight (average 600 ± 50 Kg) and stage of lactation (90 ± 20 d) were randomly selected for this trial. Four treatment groups (10 buffaloes per group) with different doses of MLF included; control (0 g/d), MLF15 (15 g/d), MLF30 (30 g/d), and MLF45 (45 g/d). Buffaloes were fed with total mix ration consisting of grass (*Pennisetum purpureum schum*), brewery's grain and concentrate mixture for 5 weeks. Meteorological data including ambient temperature and relative humidity were recorded using the online dust monitoring system to calculate temperature-humidity index (THI). After 1 week of the adaptation, milk yield was recorded daily while physiological parameters (respiratory rate, rectal, and body surface temperature), and milk composition were measured weekly. At the end of the trial, blood samples were collected to analyze serum metabolic hormones including estradiol (E2), growth hormone (GH), prolactin (PRL), Tri-iodothyronine (T3), and Thyroxine (T4). Moreover, serum heat shock proteins (HSP), antioxidants enzymes including malondialdehyde (MDA), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) and blood biochemical indices were also analyzed. Results revealed a decrease ($P = 0.012$) in serum MDA level while increasing ($P < 0.01$) the HSP and serum GHS-Px contents in supplemented buffaloes. Treatment showed a linear and quadratic decrease ($p = 0.001$) in the serum T-AOC while reducing CAT contents linearly ($p = 0.012$) as compared to the control. However, no effect of treatment on serum SOD content was observed. Treatment resulted a linear increase ($p = 0.001$) in serum GH and PRL hormones while increasing serum E2 levels linearly ($P < 0.001$) and quadratically ($P = 0.025$). Treatment increased ($p = 0.038$) the daily milk yield as compared to the control. However, increase ($P < 0.05$) in serum T3 and T4 contents, fat corrected milk (4%) and milk protein (%) was observed only in MLF45. Moreover, we observed no change in serum biochemical indices except insulin which linearly increased ($p = 0.002$) in MLF45. Our findings indicated that MLF at 45 g per day is an appropriate level to enhance milk performance and alleviate heat stress in buffaloes.

Keywords: mulberry leaf flavonoids, milk yield, antioxidant enzymes, metabolic hormones, heat shock proteins, heat stress

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INTRODUCTION

Flavonoids belong to a diverse group of plant polyphenols that are widely distributed in different plant species and possess a wide range of biological and pharmacological activities. Most of the plant species used as animal fodders are rich sources of flavonoids along with other polyphenolic contents (1). Due to excellent biological properties, flavonoids are considered efficient feed supplements for livestock to enhance performance (growth and development) and quality of animal products (2). Flavonoids from plant species of genus *Morus* (commonly known as mulberry) which belongs to the family *Moraceae* are famous for their antioxidant potential. The genus *Morus* contains about 68 species and the majority of them are cultivated in Asia (3). China has the largest area of mulberry cultivation (626,000 ha) followed by India (with nearly 280,000 ha). Several other countries [e.g., Thailand and Brazil (ca. 35,000 ha)] still have some mulberry production but on a much smaller scale (4). Owing to its easy propagation and excellent growth characteristics, it can yield leaf biomass of about 25–30 tones/ha/year with a quite less harvesting interval of about 9 to 10 weeks (5). Mulberry leaves contain rich protein content (14–30%) with high *in vivo* dry matter digestibility (75–85%) coupled with a quite luring palatability due to their succulent nature (6). Traditionally, mulberry foliage has been used as alternate forage for livestock in China, mainly due to their rich nutrient profile and flavonoid contents (7). Feeding of mulberry leaves has shown to increase fiber degradation and utilization leading to enhanced milk production in ruminants (8). Moreover, mulberry leaves have shown potent antioxidant and anti-inflammatory properties owing to their rich flavonoid contents (9).

Mulberry leaf flavonoids (MLF) are of great importance due to their excellent antioxidant, biological, and pharmacological activities (10, 11). Major flavonoids in mulberry leaf include isoquercitrin, astragalin, kaempferol, quercetin, and rutin (12). Recent studies have revealed the potential of mulberry-derived flavonoids to effectively improve or sustain animal performance and health (13, 14). The most promising activities of flavonoids in addition to antioxidants; are their potential to modulate different metabolic pathways especially those linked with energy homeostasis in the body. Due to structural homology with estrogenic hormones, flavonoids exhibit similar anabolic functions through modulation of key lipid and carbohydrate metabolic pathways (15, 16). Mulberry leaf extract has shown to upregulate the activities of glycolytic enzymes while downregulating gluconeogenic enzymes through modulation of gene and transcriptional factors involved in glucose homeostasis in the liver of mice (17). Studies have also shown that hydro-ethanolic extract of mulberry leaves significantly reduced the level of lipid peroxidation and adipocyte size in the liver. Moreover, combined mulberry leaf and fruit extract significantly reduced the obesity-related inflammation and oxidative stress in the high-fat diet obesity model (18).

Flavonoids also possess a remarkable ability to enhance non-specific immunity while reducing oxidative stress by promoting the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) while lowering the MDA level (19). This activity of flavonoids is mainly attributed to their action as a reducing agent and a hydrogen donor to scavenge reactive oxygen species (ROS) efficiently by removing the peroxide and superoxide radicals (19). Supplementation of MLF significantly improved overall antioxidant capacity while reducing the oxidative stress in *E. coli* challenged pre-weaning calves (20).

Mulberry leaf flavonoids have been extensively studied in mice and monogastric animal models, but studies on ruminants especially dairy animals, are limited. The MLF have also shown to increase the apparent digestibility of organic matter (OM) and neutral detergent fiber (NDF), while reducing methane emission in sheep (7). We hypothesized that owing to their effective antioxidant activity, MLF can effectively reduce the oxidative stress posed by the hot and humid climate of southern China. Moreover, their homology with anabolic steroids can enhance the secretion of metabolic hormones which can subsequently affect lactogenesis (21). The alleviation of oxidative stress coupled with increased secretion of metabolic hormones can synergistically lead to desirable effects on antioxidant status, nutrient metabolism, and milk production performance of buffaloes. Therefore, this study was designed to evaluate the effect of MLF supplementation on antioxidant parameters, metabolic hormones, and milk production of buffaloes under the hot and humid climate of South China.

MATERIALS AND METHODS

Materials

Extract of mulberry (*Morus alba*) leaves (flavonoids 5%) was purchased from Xi'an Feida Biotechnology Co. Ltd., Xi'an, China. The commercial MLF extract mainly consisted of flavones (65.0%), flavonols (20.0%), and other polyphenols (15.0%).

Geographical Location and Environmental Conditions

This study was conducted from June to July 2019 at the Guangxi Buffalo Research Institute, located in Nanning, South China (N 22° 53′22.59″N, E 108°21′51.19″E; 122 meters above sea level). We used an online dust monitoring system (Shenzhen Greenford Environmental Technology Co., Ltd.) to record weather data in real-time, mainly including air temperature (AT, °C) and relative humidity (RH, %), with an interval of 30 min, at an installation height buffalo's body. Environmental variables were recorded twice daily in the morning (at 8.00 A.M) and afternoon (at 2.30 P.M) during the study period (**Figure 1**). Average daily temperature and relative humidity were used to calculate Temperature Humidity Index (THI) using the following formula proposed by Thom (22);

$$\mathrm{THI} = \mathrm{AT} + 0.36\,\mathrm{DPT} + 41.5$$

Where AT is the air temperature and DPT is the dew point temperature of the buffalo shed.

Animals, Diets, and Experimental Design

All procedures used in this experiment were approved by the Ethics committee of the Chinese Academy of Agriculture

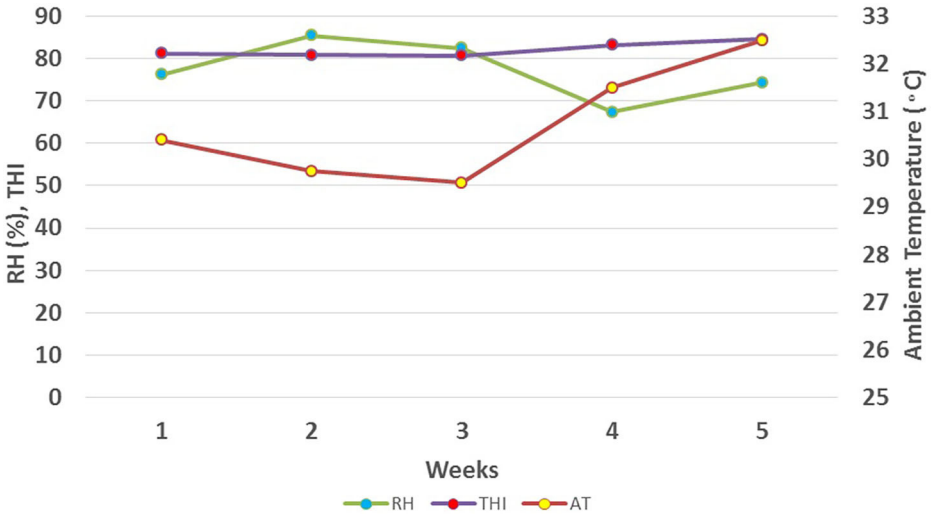

FIGURE 1 | Average weekly ambient temperature, relative humidity, and THI during study period.

Sciences, Guangxi Buffalo Research Institute, China. Forty multiparous Murrah buffaloes (4 ± 1 lactations) with an average body weight of 600 ± 50 Kg with the almost same stage of lactation (90 ± 20 days) were randomly selected for this trial. Three levels of MLF were compared in a complete randomized design to evaluate their effect on serum metabolites, stress proteins, antioxidant parameters, and milk production performance of Murrah buffaloes. Four treatment groups (10 buffaloes per group) with different doses of MLF included; MLF15 (15 g/d/head), MLF30 (30 g/d/head), MLF45 (40 g/d/head), and control group (0 g/d/head). Buffaloes were fed with total mix ration (TMR) consisting of grass (*Pennisetum purpureum schum*), brewery's grain and concentrate mixture for 5 weeks. The analysis of crude protein in TMR was performed according to AOAC procedures (23). The TMR samples were also analyzed for neutral detergent fiber (NDF) and acid detergent fiber (ADF) as described previously (24) using an ANKOM2000 Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA). Neutral detergent fiber content was analyzed with heat-stable α-amylase and sodium sulfite per sample in the neutral detergent solution. The NDF and ADF were expressed inclusive of residual ash. The gross energy (GE) of rations was determined by a bomb calorimeter (PARR Calorimeter, USA). Details of the chemical composition of the experimental diet are given in **Table 1**.

TABLE 1 | Composition and nutrient levels of basal diets (air-dry basis, %).

Items	Content
Ingredients	
Grass (*Pennisetum purpureum schum)*	32.82
Brewer's grains	48.28
Corn	9.44
Wheat bran	3.98
Soybean meal	3.03
Limestone	0.26
NaCl	0.37
$CaHPO_4$	0.32
$NaHCO_3$	0.42
Premix[a]	0.18
Total	100
Nutrient level[b]	
CP	15.23
NDF	39.57
ADF	27.98
Gross energy (MJ/kg)	16.26
Ash	9.21

[a] *The premix provided the following per kg of diets:Vitamin E 3,000 IU, Vitamin D 150 000 IU, Vitamin A 500 000 IU, Cu 1.3 g, Fe 4.0 g, Mn 3.0 g, I 80 mg, Zn6.0 g, Co 80 mg, Se 50 mg.*

[b] *Measured values.*

Animal Management and Recording of Physiological Parameters

All animals were managed under similar housing and management conditions. Buffaloes were housed in open-sided buffalo shed during milking time, where they were fed once in the morning and afternoon. For exercise, buffaloes were set free in an adjacent open area with a stocking density of 15 m^2/head. Free access to water was provided to all buffaloes throughout the day. Fans were installed in the buffalo shed above the animal's height to improve airflow. Buffaloes were allowed 30 min swimming time before milking (Once in each morning and afternoon). All animals were fed with a measured quantity of feed twice daily in the morning and evening before milking for *ad libitum* intake. Nutrient requirement of buffalo was calculated according to the technical standards for feeding of water buffalo in China, issued by Hunan Provincial Quality and Technical Supervision Bureau (25). It is based on the bodyweight

of animals and the average quantity of milk produced to meet the nutrient requirements of animals. Feed intake of each buffalo was measured individually by collecting and measuring residual feed daily for consecutive 7 days during the last week of the trial.

Weekly average body surface temperature (BST) was recorded on each Tuesday at 8:00 A.M and 2:30 P.M using an animal infrared thermometer from three different body sites (forehead, left chest, and abdomen). At the same time, rectal temperature (RT) was also recorded using a veterinary rectal thermometer (by inserting in the rectum for 15 s) while respiratory rate (RR) was recorded as times/min by observing thoracic movements using a stopwatch and a counter (for 2 min).

Milk Yield and Composition

Each buffalo was milked twice with milking machines and daily milk yield was recorded for all groups throughout the experimental period. The first week was given as an adaptation period to animals, after that milk yield for morning and evening was recorded daily for each buffalo, while milk samples for determination of milk composition were collected weekly. Milk composition (Milk total solids, protein, fat, and lactose) was analyzed immediately after milking for morning and evening separately using MilkoScanTM F120 (FOSS, Hillerød, Denmark). Fat corrected milk (FCM) at 4% was calculated by using the following equation (26);

$$\text{FCM}(4\%) = 0.4 \times \text{Milk yield} + 15 \times (\text{Milk Fat}/100) \times \text{Milk yield}$$

Blood Sampling and Haemtological Analysis

Blood samples were collected on the last day of the trial before morning feeding. Blood samples were taken in 10 ml evacuated tubes from the jugular vein to analyze hematological parameters. Two blood samples were collected from each buffalo; first blood was collected in a plain vacutainer tube (10 ml plain vacuum tubes) for obtaining serum, while the second blood sample was collected in a vacutainer tube containing EDTA as the anticoagulant for hematological analysis. Later on, samples were prepared (blood serum) or analyzed (whole blood samples) in the laboratory directly after receiving them. Blood samples in plain tubes were centrifuged at 3,000 rpm for 15 min, and serum was harvested according to standard methods (27). Whole blood samples were used for further analysis of hematological profile including total protein (TP), albumin (ALB), globulins (GLOB), blood urea nitrogen (BUN), and glucose (Glu) using commercial kits according to manufacturer's instructions.

Determination of Serum Heat Shock Proteins (HSP), Hormones, and Antioxidant Enzymes

Blood samples were put on ice after collection and immediately transferred to the laboratory for separation of serum. Collected serum was stored at −20°C until further analysis. Expression of serum stress proteins (HSP70 and 90) was determined using ELISA Kits. Moreover, metabolic hormones, including Estradiol (E2), Prolactin (PRL), growth hormone (GH), Triiodothyronine (T3), and Thyroxine (T4) were analyzed using ELISA Kits provided by CUSABIO BIOTECH CO., Wuhan, (China) through ELISA assay (ELISA microplate reader) according to manufacturer's instructions. Level of serum antioxidant enzymes including, T-AOC (A015), MDA (A003-1), T-SOD (A001-1), GSH-Px(A005), and CAT (A007-1-1) was determined through spectrophotometer using the Nanjing Built-in Kits (www.njjcbio.com) according to manufacturer's instructions. Average values for each treatment group are expressed as Mean ± S.E. The coefficient of variation (inter and intra-assay) of these kits was in the same range (<10%).

Statistical Analysis

Data were subjected to the one-way analysis of variance (ANOVA) in SPSS software (SPSS, 2014). Data were analyzed as a complete randomized design using the following statistical model;

$$Y_{ij} = X + T_i + A_j + e_{ij}$$

where X is the overall mean; T_i is the fixed effect of treatment (i = control, MLF15, MLF30, and MLF45); A_j is the random effect of the animal and e_{ij} is the random error term.

Treatment means were compared using Duncan's multiple range test. Moreover, polynomial contrasts (linear and quadratic) were measured to determine the dose-dependent response of MLF supplementation. Significant effects of the treatment were declared at $p < 0.05$.

RESULTS

Meteorological Data

Results revealed that the average ambient temperature was 30.73°C, with a range of 27.5–35°C. Relative humidity ranged from 59 to 88 % with an average of 77%. The temperature-humidity index showed a variation from a minimum value of 78 to a maximum value of 87 with, an average value of 82 during 5 weeks of the study period (**Figure 1**).

Effect of Treatment on Physiological and Serum Biochemical Parameters

Results revealed no significant effect of supplementation of MLF on physiological parameters, including body surface temperate and respiratory rate (**Table 2**). However, significantly higher rectal temperature was observed in supplemented groups (MLF15 and 45) as compared to the control group (38.47 to 38.50 vs. 38.31). Our study revealed no effect of treatment on serum biochemical parameters including TP, ALB, GLB, BUN, and glucose in lactating buffaloes (**Table 2**). However, MLF45 linearly increased ($p = 0.002$) the insulin level as compared to the control.

Effect of MLF on Serum Antioxidant Parameters

Supplementation of MLF revealed no effect on serum SOD levels in lactating buffaloes as compared to the control group (**Table 2**).

TABLE 2 | Effect of MLF on the physiological, serum biochemical, and antioxidant parameters of buffalo.

Parameter	Control	MLF15	MLF30	MLF45	SEM	*P*-Value		
						Treatment	Linear	Quadratic
Physiological parameters								
Body surface temperature (°C)	33.75	33.85	33.82	34.15	0.538	0.956	0.642	0.833
Rectal temperature (°C)	38.31^{b}	38.50^{a}	38.36^{ab}	38.47^{a}	0.049	0.047	0.142	0.477
Breathing frequency (times/ min)	21.22	20.33	21.15	21.06	0.732	0.812	0.921	0.592
Serum biochemical parameters								
Total protein (g/L)	79.98	80.12	77.86	78.08	2.063	0.839	0.956	0.328
Albumin (g/L)	37.12	37.52	35.38	37.3	1.219	0.570	0.340	0.433
Globulin (g/L)	42.86	42.6	42.48	40.78	1.817	0.867	0.560	0.553
Blood urea nitrogen (mmol/L)	12.9	14.64	16.12	16.54	1.127	0.16	0.923	0.037
Glucose (mmol/L)	4.02	3.9	3.89	3.75	0.176	0.905	0.708	0.431
Insulin (ng/ml)	0.78^{b}	0.99^{b}	1.01^{b}	1.26^{a}	0.071	0.011	0.002	0.769
Serum antioxidant parameters								
SOD (U/mL)	90.92	83.53	87.31	85.83	4.404	0.065	0.576	0.521
CAT (U/mL)	7.47^{a}	5.36^{b}	5.58^{b}	5.29^{b}	0.438	0.001	0.012	0.072
T-AOC (U/ml)	3.68^{a}	2.07^{b}	1.85^{b}	2.24^{b}	0.186	0.001	0.001	0.001
MDA (nmol/ml)	5.60^{a}	3.26^{b}	4.15^{ab}	1.39^{c}	0.467	0.012	$p < 0.001$	0.665
GSH-Px (U/ml)	639.67^{a}	740.94^{b}	788.71^{b}	509.30^{c}	26.177	$p < 0.001$	0.019	$p < 0.001$

^{a,b,c}*Values with different superscripts in the same row differ significantly ($p < 0.05$).*

Treatment showed a linear and quadratic decrease ($p = 0.001$) in the serum T-AOC while reducing CAT contents linearly ($p = 0.012$) as compared to the control. Moreover, a linear decrease ($p < 0.001$) in the serum MDA levels was observed only with lower (41 %) and high (75%) levels of MLF as compared to control. The decrease in MDA levels with a medium level of MLF supplementation was not significant as compared to control. However, serum GSH-Px linearly increased ($p = 0.019$) with the first two levels of MLF (15 and 30 g/d) as compared to the control. But, the higher level of MLF (45 g/d) resulted in a quadratic decrease ($p < 0.001$) in serum GSH-Px content as compared to control and other treatment groups.

Effects of MLF on Heat Shock Proteins and Serum Metabolic Hormones

The treatment significantly affected the expression of serum heat shock proteins in buffaloes (**Table 3**). The level of HSP90 linearly increased ($p = 0.001$) with treatment as compared to the control but the higher level of MLF (45 g/d) exhibited a more pronounced effect on HSP90 levels. However, only MLF45 linearly increased ($p = 0.04$) the expression of HSP70 as compared to the control and other treatment groups. Analysis of metabolic hormones showed a linear increase ($p < 0.001$) in serum estradiol (E2) levels with MLF30 and MLF45 as compared to the other treatment groups. However, a linear increase ($p < 0.001$) in the serum prolactin levels was observed in response to MLF treatment. Similarly, growth hormone also showed a linear increase ($p = 0.001$) in response to MLF supplementation. The first two levels of MLF (15 and 30 g/d) exhibited no effect on serum T3 and T4 levels as compared to the control group. However, the higher level of MLF (45 g/d) increased the T3 ($p = 0.003$) and T4 (both linearly and quadratically, $p < 0.05$) levels as compared to other treatment groups. The MLF45 showed a more pronounced effect on T4 levels as compared to T3 (40 vs. 25% increase as compared to the control group).

Effect of MLF on Milk Yield and Composition

Results revealed a significant effect of treatment on different milk yield and composition parameters except milk lactose (**Table 4**). The higher level of MLF increased ($p = 0.038$) the daily milk yield of buffaloes as compared to the control group. A comparison amongst different levels of MLF revealed a similar increase in daily milk yield by the first two levels (MLF15 and MLF30) as compared to the control group ($p < 0.05$). However, the higher level of MLF (MLF45) showed a more obvious increase in milk yield as compared to low and medium levels. The MLF45 linearly ($p = 0.004$) and quadratically ($p = 0.009$) increased the FCM (4%) as compared to other treatment groups. The MLF45 also increased ($p = 0.02$) the milk fat (%) as compared to MLF15 but this increase together with a decrease observed in response to MLF15 and MLF30 was not significant as compared to the control. Low and medium levels of MLF showed no effect on milk protein (%) but, the high level (MLF45) increased ($p = 0.024$) the milk protein (%) as compared to the control. Milk total solids (%) decreased ($p = 0.035$) in MLF15 as compared to MLF45 and control groups.

DISCUSSION

Physiological and Serum Biochemical Parameters

Flavonoids have shown to increase the metabolic rate by activation of brown adipose tissue, leading to thermogenesis

TABLE 3 | Effect of MLF on serum heat shock proteins and metabolic hormones in lactating buffaloes.

Parameter	Control	MLF15	MLF30	MLF45	SEM	*P*-Value		
						Treatment	Linear	Quadratic
HSP90 (ng/ml)	9.39^{c}	12.21^{b}	13.87^{b}	19.76^{a}	0.828	0.001	$p < 0.001$	0.101
HSP70 (ng/ml)	1.43^{b}	1.55^{b}	1.52^{b}	1.96^{a}	0.143	0.000	0.040	0.294
Estradiole (E2) pg/ml	255.71^{bc}	223.04^{c}	310.80^{b}	375.84^{a}	17.721	0.002	$p < 0.001$	0.025
Prolactin (μIU/ml)	452.75^{c}	568.08^{b}	602.40^{b}	779.31^{a}	36.925	$p < 0.001$	$p < 0.001$	0.429
Growth Hormone (ng/ml)	6.16^{b}	8.16^{a}	8.20^{a}	9.27^{a}	0.381	$p < 0.001$	0.001	0.257
T3 (ng/ml)	1.30^{b}	1.28^{b}	1.18^{b}	1.63^{a}	0.126	0.003	0.153	0.099
T4 (ng/ml)	35.78^{b}	35.29^{b}	34.42^{b}	50.25^{a}	3.382	$p < 0.001$	0.023	0.042

^{a,b,c}*Values with different superscripts in the same row differ significantly ($p < 0.05$).*

TABLE 4 | Effects of MLF on milk yield and composition of buffaloes.

Parameter	Control	MLF15	MLF30	MLF45	SEM	*P*-Value		
						Treatment	Linear	Quadratic
Milk yield (Kg/d)	7.11^{c}	7.35^{b}	7.37^{b}	7.67^{a}	0.177	0.038	0.064	0.869
Fat corrected milk yield (4%) Kg/d	9.75^{bc}	9.09^{c}	9.92^{b}	10.86^{a}	0.235	0.001	0.004	0.009
Milk protein (%)	4.97^{b}	4.78^{b}	4.84^{b}	5.36^{a}	0.305	0.024	0.394	0.278
Milk fat (%)	9.15^{ab}	8.25^{b}	8.98^{ab}	9.44^{a}	1.232	0.020	0.779	0.596
Total milk solids (%)	20.59^{a}	19.29^{b}	20.21^{ab}	21.34^{a}	1.467	0.035	0.642	0.431
Milk lactose (%)	5.26	5.14	5.25	5.19	0.171	0.086	0.899	0.865

^{a,b,c}*Values with different superscripts in the same row differ significantly ($p < 0.05$).*

and enhanced energy utilization. Mulberry leaf flavonoids can mediate the transcriptional signaling of different genes related to fat metabolism (28). An increase in rectal temperature in MLF15 and 45 groups indicate the effect of mulberry flavonoids on energy homeostasis in buffaloes, which is in agreement with earlier studies (29). Studies have reported that supplementation of mulberry leaf increased oxygen consumption and activity of obese mice (29). Moreover, flavonoid contents of mulberry leaf activated the brown adipose tissue (which are rich in mitochondria) through the mediation of up-regulated uncoupling protein 1 (UCP1), leading to release dietary energy as body heat (30). This heat increment may increase rectal temperature and body surface temperature in animals (29). This fact might have attributed to increased rectal temperature observed in the present study. Furthermore, activation of adipose tissues can enhance thermogenesis and energy expenditure leading to improved glucose homeostasis and insulin sensitivity (31, 32). Moreover, MLF have shown to trigger the AMPK-PGC-1α signaling pathway to improve glucose metabolism and enhance mitochondrial function to restore ATP homeostasis in skeletal muscles (33). No effect of MLF on respiratory rate and body surface temperature shows the absence of any adverse effects of MLF on the physiology of buffaloes.

No change in serum biochemical parameters was observed in the present study except insulin levels. The non-significant effect of treatment on blood metabolites is in agreement with earlier studies on beef steers fed with ensiled mulberry leaves and sun-dried mulberry pomace (34). These findings reveal that treatment with MLF showed no adverse effects on liver metabolism in buffaloes, as indicated by no change in serum metabolites. It also implies that the level of MLF used in this study is safe for lactating buffaloes as it did not show any adverse effects on downstream metabolic and physiological functions. Moreover, an increase in the serum insulin level in response to MLF45 is in agreement with earlier studies that reported enhanced insulin secretion and sensitivity in response to intra-duodenal supplementation of quercetin (a major flavonoid present in mulberry leaves) in dairy cows (35). Moreover, quercetin has also shown to increase the number of pancreatic islets, which are responsible for insulin secretion (36). In addition, quercetin enhanced the insulin release in isolated islets of Langerhans *in vitro* in a mice model (37). These findings clearly indicate that MLF can positively affect glucose metabolism and could be beneficial in regard to the metabolic adaption of high-producing dairy animals to early lactation. Additionally, optimum insulin levels and proper action are required to effectively alleviate heat stress and minimize oxidative stress damage (38, 39). Overall our findings revealed that MLF supplementation can increase insulin secretion which can subsequently improve the heat tolerance and milk performance of water buffaloes during heat stress.

Serum Antioxidant Parameters

Under normal environmental conditions (thermoneutral zone), a balance exists in the oxidant-antioxidant system to maintain body homeostasis. However, exposure to higher ambient temperature disrupts this delicate balance leading to oxidative stress. Heat stress exposes animals to an excessive load of ROS

leading to severe oxidative stress that subsequently increases lipid peroxidation and reduces immune response (40, 41). Therefore, it is crucial to maintain an oxidant-antioxidant balance to avoid the adverse effects of oxidative stress. In dairy animals, especially in lactating buffaloes, the effect of this oxidative stress is more pronounced as milk production is also a metabolic activity that generates heat. The MDA is an end product of lipid peroxidation which is commonly used as a biomarker to indicate the degree of oxidative stress and level of free radicals (42). Under oxidative stress conditions, the defense system of a body fails to scavenge a large number of free radicals in time due to reduced activities of antioxidant enzymes (CAT, SOD, GSH-Px) under chronic heat stress (43). Therefore, levels of MDA and antioxidant enzymes in ruminants are commonly used as physiological indicators to determine the degree of oxidative and heat stress (44).

In the present study, the THI value exceeded 80 during the study period which resulted in heat stress in lactating buffaloes as revealed by higher MDA levels in the control group. However, MLF significantly reduced MDA levels in treated buffaloes revealing its potent ability to alleviate oxidative stress. Remarkable decrease in MDA levels up to 75% as compared to the control indicated the useful potential of MLF to alleviate heat-induced oxidative stress. These findings are is in agreement with earlier studies reporting inhibition of ROS by mulberry flavonoids in a dose-dependent fashion (45). Even the mulberry leaf powder or their extracts have shown antioxidant, anti-inflammatory, hypolipidemic, and neuroprotective properties (9, 46, 47). A substantial decrease in MDA levels in treated groups revealed that MLF possess practical potential to decrease lipid peroxidation, as reported previously in mice model (18, 48).

The treatment showed no effect on serum SOD content in the present study. However, it significantly decreased the levels of serum T-AOC and CAT as compared to the control. Moreover, a higher GSH-Px level in MLF15 and MLF30 but lower level in MLF45 was observed as compared to the control. Studies have reported that oxidative stress induces the expression and activity of GSH-Px, hence the higher level of GSH-Px indicates more severe heat-induced oxidative stress (49). A lower level of GSH-Px observed in buffaloes supplemented with a higher dose of MLF (45 g/d) reveals the optimum level of flavonoids required to adequately scavenge free ROS ultimately leading to reduced oxidative stress and subsequent decreased activity of GSH-Px. The decrease in the activity of serum T-AOC and CAT in buffaloes supplemented with MLF revealed a relatively lower level of oxidative stress. This is in agreement with earlier studies on pre-weaning calves supplemented with MLF (20). Moreover, treatment with flavonoids has shown to enhance antioxidant capacity, improve non-specific immunity, and alleviate oxidative stress by increasing SOD and GSH-Px activity while decreasing the MDA concentration (19). It is mainly attributed to the ability of flavonoids to act as reducing agents and hydrogen donors to neutralize ROS and remove hydrogen peroxide and superoxide ions (19). The findings of the present experiment support the earlier studies about the dual functionality of MLF to alleviate oxidative stress; (1) by directly interacting with ROS (2) by increasing the activity of antioxidant enzymes.

Heat Shock Proteins and Metabolic Hormones

Heat shock proteins are well-known for their ability to mediate heat stress and considered as cellular thermometers. Especially the 70-kDa and 90-kDa heat shock proteins (HSP70 and 90) play a key role in thermo-tolerance. These proteins function as molecular chaperons and have significant roles in cellular thermotolerance, apoptosis, immune-modulation, and heat stress (50). Under oxidative stress conditions, the cellular expression of HSP is up-regulated to minimize the accumulation of denatured or abnormal proteins. The HSP are ubiquitously essential to prevent cell damage and enhance the ability to withstand oxidative and thermal stress (51).

In the present study, MLF significantly increased the serum HSP90 content as compared to the control group. However, a higher HSP90 level was observed in MLF45 while the other two treatment doses showed the same levels. Moreover, lower and medium levels of MLF (15 and 30 g/d) showed no effect on serum HSP70 content but a high level of MLF (40 g/d) significantly increased HSP70 content as compared to the control. These findings revealed a variable effective dose of MLF required to enhance the expression of HSP in buffaloes under heat stress conditions. Earlier studies in mice model have reported that quercetin can effectively upregulate the expression of the HSP (especially HSP70) through mediating the ERK/PPARγ signaling pathways (52). Moreover, the extent of such effects induced by flavonoids on HSP was dependent on the molecular weight (family) of HSP (52). This may be attributed to the fact that we observed slightly variable effects of MLF on the expression of serum HSP70 and 90.

Dietary flavonoids not only act as potent antioxidants but also regulate various signaling pathways to resist heat-induced oxidative stress at the cellular level (53). Moreover, they also possess the ability to enhance the absorption and utilization of dietary nutrients, immune response, and the development of mammary glands as well as lactation performance in animals (54, 55). In the present study, supplementation of MLF enhanced the concentration of serum metabolic hormones including E2, GH, and PRL. This may be attributed to the fact that the molecular structure of flavonoids resembles anabolic steroid hormones (phytoestrogens), which enables them to regulate the secretion of the different endocrine hormones by mediating hypothalamus-pituitary-axis (21). Owing to structural similarities of flavonoids with natural estrogen hormone along with other steroid hormones and their antagonists (56), they possess a great affinity for endoplasm reticulum and subsequent ability to mediate gene expression like estrogens, albeit at a lower affinity (57).

In the present study, a higher dose of MLF (45 g/day) showed more pronounced effects on the secretion of E2, GH, and PRL hormones as compared to low and medium levels of supplementation. We observed an increase of 46, 72, and 50 % in serum concentration of E2, GH, and PRL, respectively, in MLF45 as compared to the control group. The lower and medium levels of MLF (MLF15 and MLF30) had no effect on serum T3 and T4 contents. However, a substantial increase in serum T3 (25%) and

T4 (40%) contents was observed with a higher dose of MLF (45 g/d) in buffaloes. It reveals that the effect of MLF on endocrine physiology is dose-dependent which is in agreement with earlier studies (45, 58).

Milk Yield and Composition

Due to rich nutrient profile, flavonoid contents, and abundant availability, mulberry leaves have been considered as alternate forage for ruminants especially in China (7). Studies have shown that ensiled mulberry leaves and sun-dried mulberry pomace are also potential feed ingredients for animals (59, 60). Mulberry leaf flavonoids have shown to improve the feed intake and growth performance in ruminants. However, no study has explored the effect of MLF on milk yield and composition in dairy cattle and buffalo. Therefore, we will compare our findings with studies supplementing flavonoids from different sources. Our findings revealed a significant increase in the daily milk yield of buffaloes in response to all levels of MLF supplementation as compared to the control group. However, this increase was more pronounced in MLF45 as the other two groups did not show a significant difference between each other. Similarly, FCM (4%) and milk protein (%) was also significantly higher in MLF45 as compared to other treatment and control groups. Similar findings have been reported earlier regarding the increase in daily milk and protein yield and energy corrected milk in response to supplementation of flavonoid-rich diet containing grape seed or grape marc meal extract or green tea or Curcuma in dairy cattle (61, 62).

In the present study, the treatment showed a significant increase in milk fat and protein (%) only with a higher dose of MLF (45 g/d) as compared to the control. Increased milk protein content has been observed in an earlier study in dairy cows supplemented with quercetin (35). In addition, our findings were consistent with higher propionate and total VFA contents observed in earlier studies involving supplementation of MLF in Holstein calves (63). These desirable changes in rumen fermentation may be attributed to the favorable effect of MLF on major cellulolytic bacteria (like *Ruminococcus albus)* as supplementation of ensiled mulberry leaves in the diet of fattening steers showed a significant increase in *R. albus* (60). Such changes in gut bacteria can potentially increase cellulose degradation leading to better rumen kinetics and higher VFA yield. Moreover, diet containing ensiled mulberry leaves and mulberry fruit pomace has shown to increase the relative abundance of amylolytic bacteria (particularly *S. bovis*, and *Ruminobacter amylophilus*) which can positively influence starch degradation in the upper gut and consequently increase the glucose content of the intestine (60). It has also been suggested that mulberry leave and fruit pomace can produce more fermentable glucose in the gut and also positively influence protein utilization by microorganisms subsequently leading to better energetic efficiency in ruminants (59, 60). Moreover, flavonoids (like quercetin) have shown to decrease total protozoa and methanogens to significantly decrease *in vitro* methane production without adversely affecting rumen microbial fermentation (64). These findings collectively suggest that MLF can potentially modulate rumen microbiome to mediate fermentation kinetics subsequently leading to better nutrient utilization and performance in ruminants. It is quite pertinent to mention that in the present study, MLF substantially decreased the abundance of *Prevotella* in the rumen of buffalo (data not presented) in MLF45 as compared to MLF15 and the control. It is well-established that *Prevotella* has a negative correlation with DMI and milk fat content (65, 66). Therefore, a reduction in the abundance of *Prevotella* in MLF45 was well-associated with higher milk fat (%). Moreover, Prevotella species are more specialized for protein degradation, peptide fermentation, and their uptake in the rumen (67). Its reduction in the rumen may have increased the amount of rumen bypass protein leading to enhanced duodenal protein supply that subsequently affected milk protein contents. These findings are in agreement with earlier study reporting reduction of dietary protein degradation under *in vitro* conditions when ryegrass grass was supplemented with rutin (68). In addition, studies have reported that lower *Prevotella* abundance has been observed in high producing cows as compared to low producing dairy cows (69). It is the most likely reason for higher milk yield and protein (%) observed in MLF45 as compared to other treatment groups.

The present study revealed that MLF at 45 g per day is an appropriate dose for supplementation in buffaloes to enhance milk production performance. The dose-dependent effect of MLF observed in the present study is also consistent with earlier studies reporting the higher average daily gain in calves fed with a high level of MLF as compared to lower levels (63, 70, 71). These studies suggested that alleviation of oxidative stress by MLF can impart beneficial effects on animal health and performance as observed on the average daily gain in calves previously. The desirable effects of MLF on lactation performance observed in the present study were well-associated with levels of serum GH and PRL. The galactopoietic effects of GH are well-established as its level is positively associated with milk yield in dairy cattle (72–74). The galactopoietic effects of growth hormone are mediated via increased secretion of GH-releasing factor and insulin-like growth factor-I (IGF-I) in dairy cows (74, 75). Studies have suggested that GH stimulates milk production by partitioning nutrients from adipose tissue and muscle, increasing blood flow to the mammary gland, increasing feed intake, and reducing whole-body amino acid oxidation and urinary nitrogen loss (76). Many studies have shown that the positive effects of GH on milk yield in dairy animals are related to an increase in the proliferation and activity of mammary epithelial cells (77, 78). It may be mediated either through the direct effect of GH on the mammary gland or an indirect effect via increased secretion of IGF-1 (79, 80). Moreover, GH also modulates the ribosomal protein S6 phosphorylation leading to enhanced protein synthesis in the mammary gland ultimately increasing milk protein yield (81, 82). On the other hand, PRL also plays an important regulatory function in mammary gland development, milk secretion, and expression of milk proteins (83–85) and have shown galactopoietic effects in dairy ruminants (86). Moreover, increasing the PRL secretion by injecting a dopamine antagonist (domperidone) was found to increase milk production (87). In addition, more apoptosis was found in the mammary glands of

cows when PRL secretion was inhibited (88), suggesting that PRL is a survival factor for mammary cells and important mediator for lactation persistency. Keeping in view of the above-mentioned facts, it is suggested that a more pronounced effect of a higher dose of MLF on milk yield and milk protein (%) observed in the present study is partly attributed to the higher level of GH and PRL found in this group. In addition, higher insulin levels observed in MLF45 were well-associated with higher milk yield, FCM, and milk protein in the present study. Our findings are in agreement with earlier studies as supplementation of quercetin has shown to increase milk protein (%) in dairy cows that was believed to be associated with plasma insulin concentration via the GH-IGF axis (35, 89).

Mulberry leaf flavonoids have shown to increase the nutrient digestibility, dietary metabolizable energy, and rumen fermentation in pre and post-weaning calves (70, 90). The desirable effects of MLF on lactation performance observed in the present study may be attributed to the increased secretion of metabolic hormones and modulation of the insulin/IGF-1 signaling pathway (91). Moreover, flavonoids also possess excellent antioxidant activity, which leads to the restoration of physiological homeostasis at a cellular level like maintaining insulin levels. An increase in insulin secretion and subsequent acceleration in oxidative degradation of sugars eventually leads to enhanced feed intake and positive energy balance under heat stress conditions (92). Moreover, MLF possess functional characteristics like modulation of expression and activities of several enzymes involved in lipid and carbohydrate metabolism, which can ultimately affect animal performance to a greater extent (15, 16). Many studies have also provided the convincing evidence that flavonoids possess the therapeutic potential to improve metabolic parameters possibly through modulating peroxisome proliferator-activated receptor (PPAR)-γ, which plays a crucial role in the regulation of fatty acid and glucose metabolism (93, 94). Furthermore, mulberry leaves have also shown to maintain optimum conditions for rumen fermentation to enhance nutrient digestion and utilization in beef cattle (92). Dietary supplementation of MLF has also been reported to enhance the digestibility of organic matter and NDF in sheep (7). So, all these desirable effects can synergistically contribute to the overall beneficial outcomes in terms of better lactation performance in dairy animals as observed in this study.

According to the best of our knowledge, it is the first study to reveal the excellent antioxidant potential of MLF to alleviate heat stress in lactating buffaloes managed under the tropical environment. The increase observed in daily milk yield, FCM, and milk protein content in response to MLF45 is economically important and indicates the practical feasibility of MLF supplementation in dairy production systems particularly under heat stress conditions. The outcome of the present study suggested that alleviation of oxidative stress significantly contributes to the beneficial effects on metabolic status and lactation performance under a hot and humid climate.

CONCLUSIONS

Dietary supplementation of MLF significantly decreased the oxidative stress marker (MDA) while increasing the serum heat shock proteins, GHS-Px, and insulin contents. However, the treatment decreased T-AOC and CAT while no effect on serum SOD contents was observed owing to reduced oxidative stress in buffaloes. Our study revealed the dose-dependent effect of MLF on serum metabolic hormones (E2, PRL, GH, T3, and T4), daily milk yield, FCM (4%), and milk protein (%) in Murrah buffaloes. The present study concluded that MLF at 45 g per day is the most appropriate dose for supplementation in lactating buffaloes to enhance lactation performance and alleviation of heat-induced oxidative stress during the summer season. However, studies on a larger cohort are required to provide mechanistic insights on the role of MLF on alleviation of oxidative stress, modulation of rumen microbiome and hypothalamus-pituitary axis to mediate metabolic functions and performance in lactating buffaloes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics committee of the Chinese Academy of Agriculture Sciences, Guangxi Buffalo Research Institute, China.

AUTHOR CONTRIBUTIONS

CY: conceptualization, funding acquisition, supervision, and validation. ML and FH: data curation. FH and ZT: formal analysis. ML: investigation. ML, LP, KP, LL, and XL: methodology. XL and CY: project administration. ZT, FX, and CY: resources. FH: writing—original draft. FH, ML, and CY: writing—review & editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Invited Review: Glucosinolates Might Result in Low Methane Emissions From Ruminants Fed Brassica Forages

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Methane is formed from the microbial degradation of feeds in the digestive tract in ruminants. Methane emissions from ruminants not only result in a loss of feed energy but also contribute to global warming. Previous studies showed that brassica forages, such as forage rape, lead to less methane emitted per unit of dry matter intake than grass-based forages. Differences in rumen pH are proposed to partly explain these low emissions. Rumen microbial community differences are also observed, but the causes of these are unknown, although altered digesta flow has been proposed. This paper proposes a new mechanism underlying the lower methane emissions from sheep fed brassica forages. It is reported that feeding brassica forages to sheep can increase the concentration of free triiodothyronine (FT_3) in serum, while the intramuscular injection of FT_3 into sheep can reduce the mean retention time of digesta in the rumen. The short retention time of digesta is associated with low methane production. Glucosinolates (GSLs) are chemical components widely present in plants of the genus *Brassica*. After ruminants consume brassica forages, GSLs are broken down in the rumen. We hypothesize that GSLs or their breakdown products are absorbed into the blood and then may stimulate the secretion of thyroid hormone FT_3 in ruminants, and the altered thyroid hormone concentration may change rumen physiology. As a consequence, the mean retention time of digesta in the rumen would be altered, resulting in a decrease in methane emissions. This hypothesis on mitigation mechanism is based on the manipulation of animal physiological parameters, which, if proven, will then support the expansion of this research area.

Keywords: digesta retention time, free triiodothyronine, greenhouse gas, physiological parameters, plant secondary compounds, rumen

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INTRODUCTION

Methane (CH_4) is an important greenhouse gas, with a global warming potential of 28 times more than carbon dioxide (1). Agriculture accounts for 62% of CH_4 emissions from anthropogenic activities, while ruminants account for 58% of the CH_4 emissions from agriculture (2). As a result, enteric CH_4 emissions are the single largest source of anthropogenic CH_4 contributing to the

global greenhouse gas emissions (3). Methane emissions also cause energy losses in livestock, which account for 3.9–10.7% of the metabolic energy ingested, resulting in less efficient energy utilization by the animal (4). Reducing CH_4 emissions, therefore, has the potential to improve feed conversion efficiency (5). Thus, mitigation of CH_4 emissions helps not only environmental protection but also has substantial economic benefits to promote sustainable development of animal husbandry (6, 7). For this reason, mitigation of CH_4 emissions from ruminants has become a highly active research topic in animal husbandry.

Methane is formed in the process of the rumen microbial degradation of feed in ruminants. The approaches to the mitigation of CH_4 emissions include inhibiting methanogens with inhibitors (8) or a vaccine, modifying microbial activity in the rumen with electron acceptors, ionophores (9), or dietary manipulation (10), and breeding for low-CH_4 livestock (11). Among these approaches, the use of brassica forages to mitigate CH_4 emissions is a feasible method that does not change farming systems, increase production costs or result in artificial chemical residues.

The purpose of this review is to summarize literature reports on the use of brassica forage to mitigate CH_4 emissions, analyze possible mitigation mechanisms, and highlight the possible role of glucosinolates, which are characteristic substances in brassica forages, in reducing CH_4 emissions.

RUMINANTS FED FORAGE BRASSICA EMIT LOW METHANE

Brassica forage crops including kale (*Brassica oleracea*), turnip (*Brassica campestris*), forage rape (*Brassica napus*), and swede (*Brassica napus* ssp. *rapifera*) are annual plants, grown worldwide to provide ruminants feed, in many cases during the period when forage supply is limited in quantity or quality (12). These crops can grow in winter, but forage rape and bulb and leafy turnips can also grow in summer. These crops have high water-use efficiency and thus are suitable to grow in conditions of limited water resources (13). They have a short growth period (14), being easy to grow, with the ability to be intercropped with legumes (15).

Forage brassica crops have the characteristics of having a high yield, typically 2–8 t dry matter (DM)/ha for leafy turnip, 3–10 t/ha for forage rape, 2–12 t/ha for turnips, 5–20 t/ha for kale and 5–20 t/ha for swedes (16). The leaves and stems of kale, leafy turnip, and forage rape are used for feed, while swede and bulb turnip are root brassicas with both leaves and bulbs being used. The chemical composition of brassica forages varies greatly among species and within a species mainly due to the difference in the ratio of leaves to bulbs or to stems (17). Compared with perennial ryegrass (*Lolium perenne*), brassica forages contain less neutral detergent fiber (NDF) and more readily fermentable carbohydrate (12, 18). The content of NDF is 271–328 g/kg DM for kale, 180–240 g/kg DM for forage rape and turnip, and 165–196 g/kg DM for swede (12, 17, 18, 18–20), while the content of readily fermentable carbohydrates was 253 g/kg DM for kale, 285 g/kg DM for forage rape, 334 g/kg DM for turnip, and 370 g/kg DM for swede (18). Among readily fermentable carbohydrates, the content of pectin is about 69–94 g/kg DM (18), the content of sugars (raffinose, sucrose, glucose, fructose) and starch is 205 g/kg DM for kale, 138 g/kg DM for forage rape, 194 g/kg DM for turnip, and 283 g/kg DM for swede (17). The content of crude protein is 130–162 g/kg DM for bulb brassicas and 167–193 g/kg DM for leafy brassicas (18). The low content of NDF and the high content of readily fermentable carbohydrates lead to a high ruminal degradation rate (21, 22), a high DM digestibility (810–890 g/kg), and a high metabolizable energy content (12.1–14.1 MJ/kg DM) and thus have a high feeding value for ruminants (12, 18). As a result, these crops have been applied in farming practice in sheep (23, 24), beef cattle (25), dairy cows (25–27), and deer (12).

Research on the use of brassica forages for the mitigation of CH_4 emissions from ruminants began in New Zealand. Sun et al. (18) reported for the first time that four common forage brassica crops in New Zealand kale, turnip, rape, and swede fed to sheep in winter resulted in lower CH_4 yield (CH_4 emissions per unit of DM intake) by 10, 6, 25, and 23%, respectively, compared with the control perennial ryegrass.

A series of animal experiments were conducted after this study (28). These experiments were conducted under various conditions including short- vs. long-term feeding (29), indoor feeding vs. grazing (30), winter vs. summer varieties (31), different brassica types (32), and primary growth vs. regrowth (32). Under these conditions, CH_4 emissions were always lower than the control perennial ryegrass-based pasture. When forage rape was mixed with perennial ryegrass to form mixed diets with gradual inclusion levels for sheep, CH_4 yields declined linearly with the increase in the proportion of forage rape in the diet (33). Heifers fed forage rape also emitted less CH_4 than those fed perennial ryegrass-based pasture (34). A study conducted in Australia showed that feeding dairy cows with brassica forage (*B. napus* cv. *Winfred*) during summer resulted in a 21% lower CH_4 yield than feeding chicory (*Cichorium intybus*) (35). It was concluded that both sheep and cattle fed different forage brassica crops in different seasons as a sole diet, or as a component of a mixed diet, under housed feeding or grazing conditions, emit low CH_4 to varying degrees, and the mitigation effect does not disappear with extended feeding.

SECONDARY METABOLITES IN FORAGE BRASSICA MAY CONTRIBUTE TO LOW METHANE EMISSIONS

The known mechanisms for the mitigation of enteric CH_4 emissions mainly include the manipulation of rumen microbiota by methods such as the addition of CH_4 inhibitors, and the manipulation of fermentation substrates of rumen microorganisms, such as altering dietary composition and providing electron acceptors (10, 36). These mechanisms cannot fully explain the low CH_4 emissions with forage brassicas.

Lower CH_4 yields from forage brassica were associated with differences in the rumen microbiota compared to perennial ryegrass, with a proposal for shifts in fermentation to more propionate and less hydrogen, resulting in less CH_4

(29). However, the factors resulting in this rumen microbial community difference are not fully understood. A multiple regression analysis of the conventional nutrients of brassica crops and CH_4 yield showed that only water content among these nutrients had a weak correlation with emissions (37). Nitrate and sulfate can be used as electron acceptors to reduce CH_4 emissions (38), but their contents in forage brassica crops vary widely (12, 39). Even the highest contents in brassica crops explain but a small proportion of the reduction in CH_4 emissions (18, 29). A meta-analysis of the relationship between rumen fermentation parameters and CH_4 yield in sheep showed that fermentation type, as indicated by the ratio of acetate to propionate and butyrate, had a limited effect (40). A low rumen pH is associated with low methanogenesis (41, 42). While the rumen pH was low in sheep fed forage rape (29), a study in which rumen pH of forage rape-fed sheep was manipulated by adding sodium carbonate did not suggest that the low CH_4 yield results totally from low rumen pH (43). The rumen microbial community of sheep fed forage rape differed greatly from that of sheep fed perennial ryegrass. For example, there were increased abundances of *Selenomonas* and *Sharpea*, whereas *Selenomonas* is known to produce acetate and propionate or lactate and *Sharpea* are lactate producers (44) and linked to low CH_4 via a proposed pathway (45). However, the importance of altered microbial community in low CH_4 emissions might be limited (29). In summary, although rumen fermentation type has a limited effect, and some factors such as conventional nutrients, nitrate and sulfate in forage brassica can be ruled out for the explanation of low CH_4 emissions with forage brassica, the reason why is still unclear.

It has been reported that chicory (46, 47) and white clover (*Trifolium repens*) (48) do not result in lower CH_4 emissions compared with perennial ryegrass-based pasture. Chicory, white clover and forage brassicas are dicotyledonous plants. The conventional nutritional composition is similar, but the secondary metabolites between them are different. The discrepancy in CH_4 emissions from these plants suggests that secondary metabolites in forage brassicas might play a role in mitigation. Glucosinolates (GSLs) and *S*-methyl-L-cysteine sulfoxide (SMCO) are two types of plant secondary metabolites which are widely present in *Brassica* plants (49, 50). Therefore, these two types of compounds should be a focus for exploring the mechanisms underlying the low CH_4 emissions with brassica forages.

SECONDARY METABOLITES IN FORAGE BRASSICAS

Concerns over GSLs and SMCO mainly due to anti-nutritional effects in animals (12, 51) and possible beneficial effects to human health (52, 53) contributed to the need for this review. Glucosinolates are a class of sulfur-containing anionic hydrophilic plant secondary metabolites whose core structure is β-D-glucose linked to a sulfonate aldoxime group (**Figure 1**), and a side chain derived from amino acids (54).

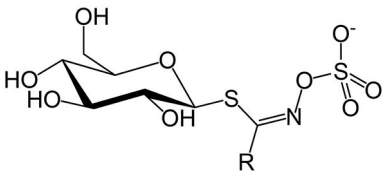

FIGURE 1 | Glucosinolate structure (the side group R varies).

Glucosinolates have no physiological activity *per se,* and after combination with β-glucosidase (also known as myrosinase), they are degraded to a variety of biologically active substances, which have toxic effects on herbivores and also function as repellents (55). Glucosinolates and β-glucosidase are present in different cells or different areas of the same cell in plants, and they react when the plant is mechanically damaged or chewed by the animal. The products of the breakdown are mainly isothiocyanate (ITC), thiocyanate, nitrile, epithionitrile and oxzolidine-2-thione, of which ITC is the most important product (**Figure 2**) (52).

There are extensive studies of GSLs in brassica vegetables and oil crops (57), but there are few studies on brassica forages. Velasco et al. (58) found that GSL profile differs greatly in rape varieties for the use as vegetables, oilseeds and feeds. Knowledge gained from brassicas for other uses cannot be applied directly to brassica crops for feed use. It has been reported that there are many types of GSLs in brassica forages, up to 18, but the contents of individual GSLs vary greatly (**Table 1**). Among them, 3–4 GSLs are predominant, accounting for more than 80% of the total content. Each brassica forage crop has its own predominant GSLs, but glucobrassicanapin is generally more than 40% of the total GSL content in forage rape, swede and turnip, and sinigrin exceeds 40% of the total GSL content in kale (18). The breakdown products of GSLs in brassica forages have been reported (59) and knowledge about them is generally derived from research results from brassica crops used for other purposes (52). Rapeseed cake contains a large amount of GSLs and the structure, breakdown products and their effects on animals have been extensively reviewed (56). Glucosinolates produce mainly ITC and nitriles in the rumen. During digestion, about 21–41% of GSLs in kale leaves are converted to nitriles, 37% for swede leaves and 50% for swede bulbs (12). Nitriles are not degraded for at least 23 h after sheep consume kale, but completely degraded within 4 h for swede bulbs and leaves.

The non-protein amino acid SMCO is about 1–2% by dry weight in brassica plants (60). The contents of SMCO vary among forage brassica species and is especially high in kale (12). The SMCO contents are also affected by fertilizer application (12), silage making (61) and harvesting (62). When plant tissues are broken, cysteine sulfoxide lyases in the vacuole are released, resulting in decomposition of SMCO into ammonia, pyruvate and methanesulphenic acid (60). Complete conversion of SMCO to dimethyl disulphide occurs in the rumen, and dimethyl

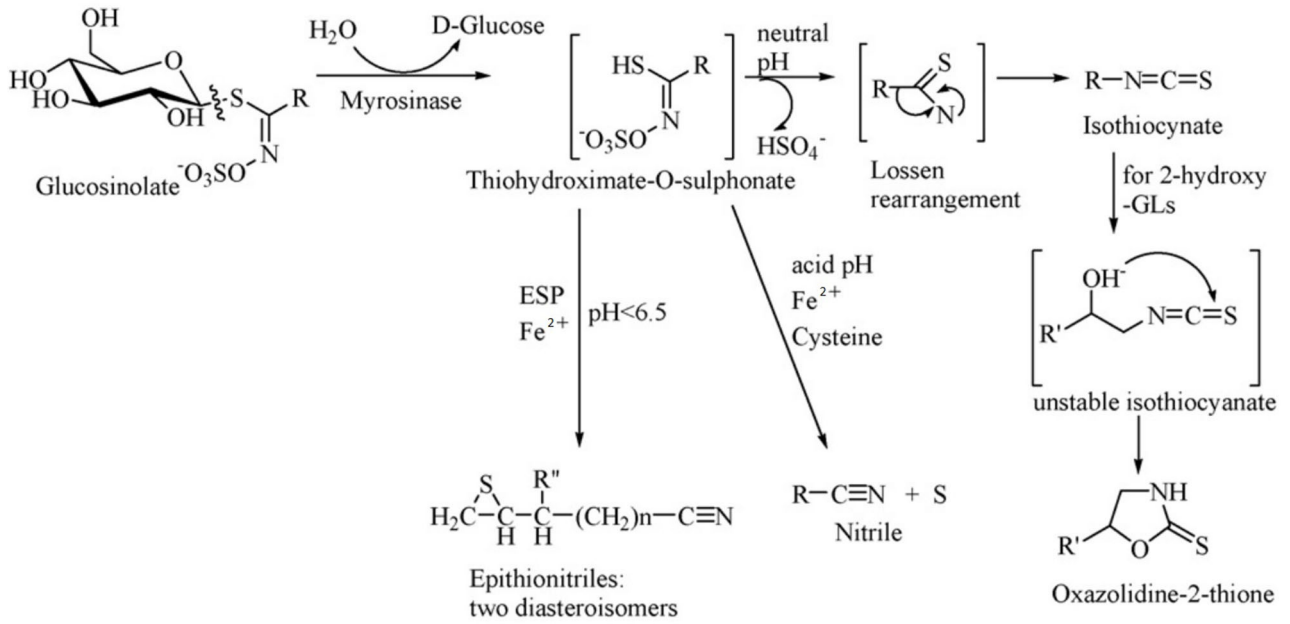

FIGURE 2 | Diagram of enzymatic hydrolysis of glucosinolates. Adapted from Tripathi and Mishra (56).

TABLE 1 | Concentration of total glucosinolates (GSLs) and proportion of individual GSLs in brassica forages[a].

Items	Kale (*Brassica oleracea*)	Rape (*B. napus*)	Swede (*B. napus*)	Turnip (*B. campestris*)
Total glucosinolate (μmol/kg dry matter)	229.3	308.4	803.8	1218.1
Proportion of individual GSLs in the total GSLs				
Sinigrin	40.6	0.0	0.0	0.0
Glucobrassicanapin	0.1	44.4	40.0	44.8
Epiprogoitrin	23.6	17.7	16.9	13.1
Gluconapin	17.5	10.5	14.0	22.2
Gluconasturtiin	0.8	3.6	14.6	6.9
Gluconapoleiferin	0.0	8.3	4.9	7.1
Glucoraphanin	8.0	0.7	0.8	0.3
Glucobrassicin	7.4	5.4	0.2	0.2
Glucoalyssin	0.3	4.7	2.4	1.0
Progoitrin	0.0	3.2	3.0	3.3
Sinalbin	1.7	1.5	0.7	0.4
Glucoiberin	0.0	0.0	1.6	0.4
Glucoerucin	nd[b]	nd	0.9	0.2
Glucotropaeolin	0.0	0.0	0.0	0.0
4-hydroxyglucobrassicin	0.0	nd	0.0	0.0
Glucobarbarin	nd	nd	0.0	0.0
Glucoraphenin	nd	nd	0.0	nd
Glucosibarin	nd	nd	0.0	nd

[a]*Adapted from Sun et al. (18).*
[b]*nd, not detected.*

disulphide inactivates proteins by combining the sulphdryl group in proteins. For example, dimethyl disulphide can reduce the content of hemoglobin, and even cause anemia, and can also affect the production of host and microbial proteins in the body. *S*-methyl-L-cysteine sulfoxide can increase ghrelin and thyroid hormones in the plasma, which can stimulate the body's

protein synthesis to replace the protein inactivated by dimethyl sulfoxide (12).

SECONDARY METABOLITES MIGHT NOT DIRECTLY INHIBIT METHANOGENS

Jayanegara et al. (63) found that *Brassica crassifolia* resulted in 25% less CH_4 emissions than grass hay in an *in vitro* batch culture study. Dillard et al. (64) studied the methanogenesis of brassica forage crops using a continuous fermentation system with half of the culture substrate as *Dactylis glomerata* and the other half as forage rape, oilseed rape (*B. napus*), turnip or annual ryegrass (*L. multflorum*). They found that CH_4 production from forage brassica crops was lower than from annual ryegrass. The conclusion was the same when the emissions were expressed as per unit of incubated organic matter, neutral detergent fiber, digestible organic matter and digestible neutral detergent fiber. Jayanegara et al. (63) and Dillard et al. (64) speculated that the plant secondary metabolites in brassica crops play a role as methanogen inhibitors in the reduction of CH_4 production. In an *in vitro* study conducted by Durmic et al. (65), a hybrid of kale and turnip (*B. napus* cv. *Winfred*) and turnip produced 30% less CH_4 in comparison with the control arrowhead clover (*Trifolium vesiculosum*), but there were great variations among different cultivars of the same forage species. Broccoli (*B. oleracea*) and a hybrid of turnip and forage rape (*B. campestris* × *B. napus*) were not significantly different from arrowhead clover in CH_4 production. The characteristic phenomenon of methanogen inhibition *in vitro* is the release and accumulation of a large amount of hydrogen (66). These studies did not measure the concentration of hydrogen emitted, which makes it difficult to determine if methanogen inhibitors were present in these feedstuffs. Sun and Pacheco (32) did not find a significant difference in methanogenesis between forage brassicas, including kale, turnip, forage rape and swede, and perennial ryegrass in an *in vitro* study, and significant emissions and accumulation of hydrogen were not observed, suggesting that no methanogen inhibitor exists in brassica forages.

Researchers have also used GSLs and their breakdown products directly to test for effects on CH_4 emission in *in vitro* rumen fermentation studies. The addition of GSLs extracted from mustard cake at doses of 0, 9, 18, 27 and 45 mg/100 mL did not adversely affect the total short-chain fatty acid (SCFA) concentration and microbial activity, but the proportion of CH_4 in the total gas production increased with the amount dosed, indicating no inhibitory effects (67). Reduced CH_4 production and hydrogen accumulation were observed when allyl isothiocyanate, a breakdown product of sinigrin, was added at doses of 48 and 96 mg/L (68) or at a dose of 75 mg/L (69) for *in vitro* incubation. Similar results were also obtained in *in vitro* studies with allyl isothiocyanate-containing mustard seeds (70) or mustard cake (71). However, the concentrations of GSL breakdown products in these studies were much higher than those that are present in brassica forages as a sole diet in natural conditions. There was no significant difference in CH_4 production between broccoli cultivars with a high or low content of GSLs as substrates for *in vitro* culture (65). In an animal study, although CH_4 emissions were not measured, the ruminal concentrations of SCFAs and the ratio of acetate to propionate did not differ in the rumen of steers fed either high- or low-GSL rapeseed (*Brassica napus* cv *Bridger* and *Dwarf Essex*) forage (72). The effects of SMCO on CH_4 emissions have not been reported. According to the literature mentioned above, GSLs, SMCO, and their breakdown products are unlikely to be methanogen inhibitors.

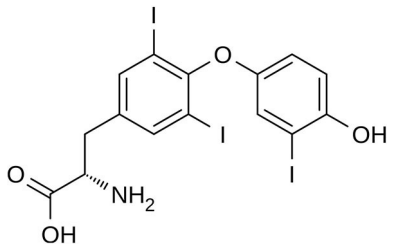

FIGURE 3 | Triiodothyronine structure.

GLUCOSINOLATES MIGHT RESULT IN LOW METHANE EMISSIONS VIA ANIMAL PHYSIOLOGICAL PARAMETERS

Effects of Secondary Metabolites in Brassica Forages on Triiodothyronine

Triiodothyronine (T_3) (**Figure 3**) and thyroxine (T_4) are two thyroid hormones produced and released by the thyroid gland (73). Thyroxine can be converted to T_3, which is three to four times more metabolically active than T_4 (74). The thyroid hormones in the blood are mainly present in the form of T_4 with a ratio of T_4 to T_3 at ∼14:1. The major fraction of the thyroid hormones is bound with transport proteins, and a small fraction is free and biologically active. Thus, the concentrations of free thyroid hormones, especially free T_3, are measured as indicators of the hormone activity.

Feeding brassica forages or diets containing GSLs and their breakdown products can affect animal thyroid function and alter thyroid hormone secretion (56, 75–79). For example, feeding turnip (*Brassica rapa* L.) and kale (*B. oleracea* L. var. *acephala* DC) to fattening lambs can increase the concentrations of T_3 and T_4 in serum (76). Feeding a diet containing a high content of GSLs to calves resulted in a quadratically increased serum T_4 concentration, although T_3 concentration remained within the normal physiological range (79). Diets contained GSLs affect thyroid function in many animal species (56), including pigs (80), mares (81), turkeys (82), hens (83), and turbots (84), suggesting that effects of GSLs on thyroid function are not unique to ruminants, but universal in a wide range of animal species.

The mechanisms of how GSLs affect thyroid hormones in ruminant animals are not clear, but it is believed to be related to iodine and selenium (74). Iodine is a component of the hormones, while deiodinases involved in the conversion of T_4

to T_3 are selenium-containing enzymes, and thus selenium is essential for T_3 production. It is recommended to supplement sheep grazing kale with iodine to lighten the effects of GSLs (75). The impacts of GSLs present in the diet can be counteracted with the supplementation of iodine or iodine plus selenium to sheep (77, 85). Iodine uptake by the thyroid can be inhibited by GSLs and their breakdown products such as goitrin and isothiocyanates (52, 86, 87). In a rat study, nitriles, another group of GSL breakdown products were considered to result in the enlargement of the thyroid (88).

Ruminal microorganisms break SMCO down into dimethyl disulfide, causing anemia (60), but activities on thyroid physiology were not reported. It is unlikely that SMCO has an effect on blood FT_3 concentration.

Effects of Free Triiodothyronine on Digesta Retention Time

Thyroid hormones are associated with digesta excretion from the rumen. Sheep exposed to a cold environment (2–5°C) had a 1.5 times greater T_3 concentration in plasma (152 vs. 62 ng/100 mL) and a 6.2 h shorter rumen mean retention time (11.8 vs. 18 h) than those exposed to a warm environment (22–25°C) (89). A greater T_3 concentration (103 vs. 21 ng/100 mL) in sheep plasma resulting from a daily injection of 0.25 mg T_3 also reduced the rumen mean retention time (17.8 vs. 20.4 h) (89). The removal of the thyroid gland from sheep housed at 22–25°C caused T_3 concentration to drop from 38 ng/100 mL to zero in plasma and the rumen mean retention time extended from 17.9 to 23.6 h (89). In a study with mature ewes by Lourenço et al. (90), it was observed that liquid rumen retention time was 18.5 vs. 26.3 h, while T_3 concentration was 83–99 vs. 59–67 ng/100 mL at high (25°C) and low (11°C) temperatures, respectively (90). When mature wethers were injected with 300 μg of FT_3 every 2 days, the blood FT_3 concentration increased from 16 to 54 ng/100 mL, and digesta retention time in the whole digestive tract was reduced by 4 h (91).

The mechanism of thyroid hormones affecting rumen physiology is unknown, but in the human body, thyroid disorders are associated with gastrointestinal dysfunction (92). It is proposed that thyroid hormones affect gut motility either directly or via a central stimulatory effect on the chemoreceptor trigger zone.

Effects of Digesta Retention Time on Methane Emissions

Pinares-Patino et al. (93) found that short digesta mean retention time, especially in the particulate phase, was associated with low CH_4 production in sheep fed alfalfa. In a study on mature ewes divergently selected for high and low CH_4 yields, a shorter mean retention time of particulate and liquid digesta was associated with low CH_4 yield (94). This association was further confirmed in a recent study by Bond et al. (95), who measured rumen digesta flow and CH_4 yield using open-circuit respiration chambers in ewes phenotypically differing in CH_4 emissions. Using simulation with mathematical models, Huhtanen et al. (96) also demonstrated that dairy cows and sheep with short digesta retention times emit less CH_4.

Low-CH_4 yielding sheep not only had smaller rumens (97), but these sheep had different rumen microbial communities compared to high-CH_4 yielding sheep (98). Detailed studies of these sheep indicated that their microbial communities were fermenting feed using different pathways that led to the observed lower CH_4 yields (45), and these differences were attributed to faster passage rates through the rumen of the low-CH_4 yield sheep (45, 99). Furthermore, a recent study integrating rumen wall transcriptome data and CH_4 phenotypes found that a set of rumen muscle genes is involved in cell junctions, which could be potential regulators of rumen digesta retention time and thus could be a molecular mechanism for the association of rumen digesta retention time with CH_4 yield in sheep (100).

Effects of Free Triiodothyronine on Methane Emissions

Elevating serum FT_3 by intramuscular injection of FT_3 in sheep can result in shorter digesta retention time and consequently reduce CH_4 yield by 8% (91). An increase in blood FT_3 concentration in sheep a result of a decrease in ambient temperature also lead to reduced digesta mean retention time and decreases CH_4 yield (101).

Hypothesis

Based on the literature reviewed here, a hypothesis is proposed that under normal farming conditions, the secondary metabolites GSLs and/or their breakdown products in brassica forage crops do not directly inhibit the growth and activity of methanogens, but increase blood FT_3 concentration in ruminants, resulting in a decrease in digesta mean retention time in the rumen, thereby reducing CH_4 emissions (**Figure 4**).

CONCLUDING REMARKS

Climate change is a topic of increasing concern in the world. Anthropogenic activities, including industrial and agricultural production, emit greenhouse gases that are the main drivers of climate change. Methane is an important greenhouse gas, and ruminal fermentation of feed is an important source of CH_4. Exploring simple, effective and low-cost approaches without side effects to mitigate CH_4 emissions from ruminants is supported by the governments of most countries. Reducing CH_4 emissions from ruminants not only helps to slow down climate change but also improves the feed energy efficiency of ruminants. Therefore, the study of the ruminant CH_4 emission mechanism is of great significance.

This article puts forward a hypothesis that the secondary metabolites of brassica forage crops GSL and its metabolites can elevate the concentration of FT_3 in ruminants and lead to a reduction in mean ruminal digesta retention time, thereby reducing CH_4 emissions. This is a new mechanism in which the mitigation of CH_4 emissions is achieved by manipulating ruminant physiological parameters and goes beyond the existing mechanisms which limit the mitigation to the manipulation of rumen microorganisms and their substrates.

If this hypothesis is confirmed, it will be a new direction for the mitigation of CH_4 emissions from ruminants and will expand research to a new field with great research value. Further

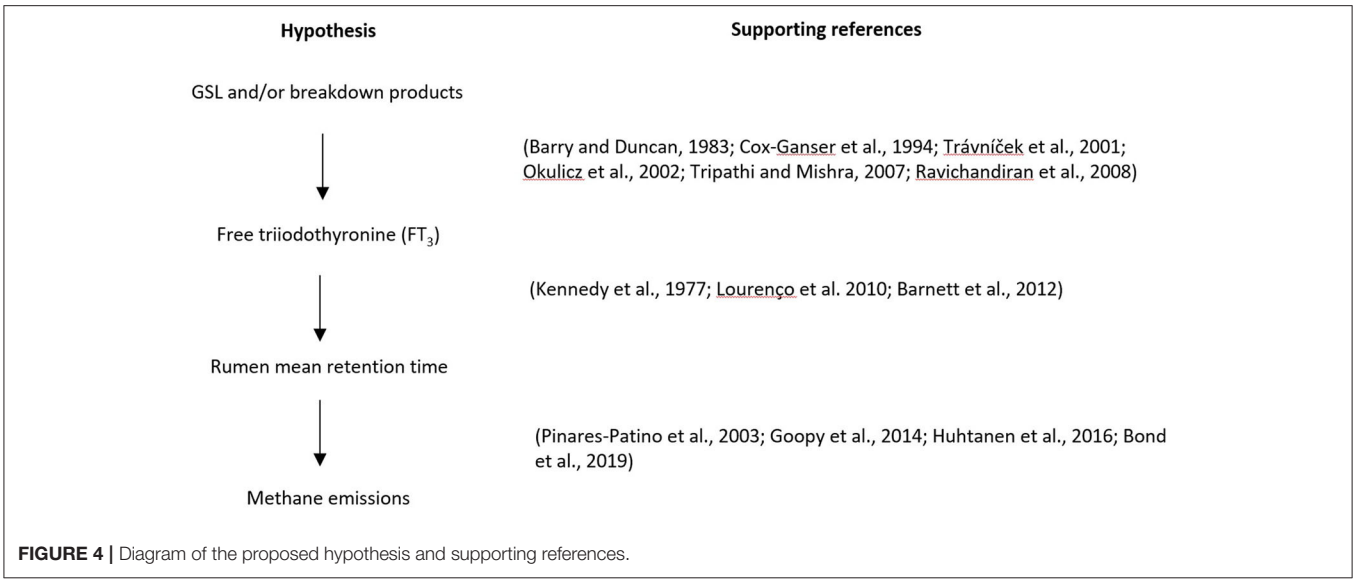

FIGURE 4 | Diagram of the proposed hypothesis and supporting references.

questions to be answered include how individual GSLs affect FT_3 and CH_4 emissions differently, what the molecular mechanism of GSLs affecting the function of the thyroid gland and the secretion of FT is, how FT_3 affect rumen digesta retention time, how FT_3 affect rumen muscle genes, etc. As brassica forages are common forages, this hypothesis is of great value in ruminant livestock methane abatement studies. Approaches to enhanced mitigation efficiency could be found by a deep understanding of these questions.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Phytogenic Additives Can Modulate Rumen Microbiome to Mediate Fermentation Kinetics and Methanogenesis Through Exploiting Diet–Microbe Interaction

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Ruminants inhabit the consortia of gut microbes that play a critical functional role in their maintenance and nourishment by enabling them to use cellulosic and non-cellulosic feed material. These gut microbes perform major physiological activities, including digestion and metabolism of dietary components, to derive energy to meet major protein (65–85%) and energy (ca 80%) requirements of the host. Owing to their contribution to digestive physiology, rumen microbes are considered one of the crucial factors affecting feed conversion efficiency in ruminants. Any change in the rumen microbiome has an imperative effect on animal physiology. Ruminal microbes are fundamentally anaerobic and produce various compounds during rumen fermentation, which are directly used by the host or other microbes. Methane (CH_4) is produced by methanogens through utilizing metabolic hydrogen during rumen fermentation. Maximizing the flow of metabolic hydrogen in the rumen away from CH_4 and toward volatile fatty acids (VFA) would increase the efficiency of ruminant production and decrease its environmental impact. Understanding of microbial diversity and rumen dynamics is not only crucial for the optimization of host efficiency but also required to mediate emission of greenhouse gases (GHGs) from ruminants. There are various strategies to modulate the rumen microbiome, mainly including dietary interventions and the use of different feed additives. Phytogenic feed additives, mainly plant secondary compounds, have been shown to modulate rumen microflora and change rumen fermentation dynamics leading to enhanced animal performance. Many *in vitro* and *in vivo* studies aimed to evaluate the use of plant secondary metabolites in ruminants have been conducted using different plants or their extract or essential oils. This review specifically aims to provide insights into dietary interactions of rumen microbes and their subsequent consequences on rumen fermentation. Moreover, a comprehensive overview of the modulation of rumen microbiome by using phytogenic compounds (essential oils, saponins, and tannins) for

manipulating rumen dynamics to mediate CH_4 emanation from livestock is presented. We have also discussed the pros and cons of each strategy along with future prospective of dietary modulation of rumen microbiome to improve the performance of ruminants while decreasing GHG emissions.

Keywords: rumen, microbiome, methane, fermentation, VFA, plant secondary metabolites

INTRODUCTION

Improving feed efficiency and livestock production is a more coveted goal in animal agriculture being sought through selective breeding, scientific management, and improvement of feed composition. Feed efficiency in ruminants mainly depends upon the quality of feed, rumen fermentation, and dynamics mediated by rumen microbiomes. The rumen in animals is inhabited by the diverse microbiome, including bacteria, protozoa, fungi, and archaea. Different factors like temperature (38–42°C), pH (5.5–7), and redox potential (250–450 mV) regulated by saliva buffering provide a specific environment for degradation of cellulolytic plant material by microbes (1).

Degradation of various feed components is being accomplished by mutual interaction of microbiota to yield mainly acetate, propionate, butyrate, hydrogen (H_2), carbon dioxide (CO_2), and ammonia (NH_3). Total VFA (75% of total amount) are the primary source of energy for the animal (2). Besides, microbial cell biomass is also utilized as the primary origin of protein and amino acids by host animals (3). The microbial ecosystem likewise produces vitamins B and K and utilizes the products of phytotoxin and mycotoxin detoxification processes (4). The ingested fiber is mainly degraded by bacteria and fungi into soluble nutrients (5). These soluble nutrients are subsequently used for the maintenance, growth, production, and reproduction of animals. During rumen fermentation of feed, some by-products are additionally produced, such as CO_2 and H_2, which are further converted into CH_4 by some methanogens like *Methanopyrales*, *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, *Methanocellales*, and *Methanosarcinales*). Some archaea (*Methanoplasmatales* or *Thermoplasmatales*) can also form CH_4 through other substrates, such as methanol and mono-, di-, and tri-methylamine (6–8). Major greenhouse gases (CH_4 and CO_2) are released during enteric fermentation from ruminants. Production of CH_4 also deprives the host animal of carbon resources and results in loss of energy (13.3 Mcal/kg CH_4), leading to poor feed efficiency (9). Maximizing the flow of metabolic hydrogen ([H]) in the rumen away from CH_4 and toward VFA would increase the efficiency of ruminant production and decrease its environmental impact. Czerkawski (10) proposed that inhibiting methanogenesis could favor microbial biomass production as an alternative [H] sink. Chalupa (11) suggested that metabolic hydrogen incorporated into excess NADH was redirected to fatty acid synthesis and fermentation end products such as lactate and ethanol, although the latter sinks were not quantitatively important (12).

Owing to its diverse physiological and metabolic functions, the rumen microbiome is considered the ultimate target to improve the energetic efficiency of animals while reducing environmental hazards like CH_4 emissions. Highly efficient animals produce less CH_4 and produce more milk, consuming less feed owing to their unique set of rumen microbiome (13). Specific physiological processes in lactating animals are correlated with specific rumen microbes owing to their unique fermentation and metabolic activities (14). The association of VFA composition with rumen bacteria has been reported in dairy cows possessing different efficiencies of production (15). Moreover, the rumen microbiome varies significantly among different animals, but intra-animal variation in microflora is quite less (16). These facts indicate the crucial role of the rumen microbiome in shaping the physiology of digestion and production in lactating animals and its potential utility for manipulation of performance and health.

Greenhouse gases (GHG) produced from ruminants have been an area of environmental concern (17, 18). Improving animal production systems must understand societal concerns and should realize the effect of such systems on the environment (19). Increasing feed efficiency to enhance animal production should also focus on CH_4 mitigation strategies to reduce GHG emissions. In this regard, identification and manipulation of the microbes associated with methanogenesis are considered a significant and most crucial step (19, 20). Methanogenesis occurs both in the rumen and hindgut, but 90% of the total CH_4 production originates from the rumen (21). A better understanding of digestive physiology and feed fermentation in rumen is necessary to ensure further improvement of production efficiency in ruminants to overcome the increasing demand for food by growing the human population. To achieve this daring task, manipulation of rumen fermentation is required to increase feed conversion efficiency while decreasing energy losses in the form of CH_4 emanations through dietary interventions (22).

Manipulation of rumen fermentation is considered as an optimization process to seek suitable conditions for maximization and/or minimization of the specific rumen fermentation pathways, depending on factors such as type and level of feeding and animal production. The basic target behind such manipulation is the alteration in ruminal microflora that can be achieved by dietary intervention and the use of additives that selectively affect rumen communities. Improvement in ruminant production is possible with the manipulation of rumen fermentation to increase total VFA and propionate production while decreasing CH_4 emission through reducing rumen methanogenesis (23). Many feed additives such as antibiotics, ionophores, and defaunating agents have been utilized to mediate rumen fermentation to improve the productivity of ruminants and reduce methanogenesis. However, most chemical additives

either are noxious to host animals or present a temporary impact on methanogenesis (24, 25). Therefore, nutritionists and microbiologists are continuously trying to explore some natural substances with anti-methanogenic activity for eco-friendly animal production by reducing CH_4 emission and its greenhouse effects (26).

We need to strengthen our understanding of diet–microbe interactions to devise dietary interventions to modulate the rumen microbiome to improve production efficiency and reduce energy losses in the form of GHG emissions. Moreover, we need to explore natural feed additives with limited or no adverse effects to manipulate rumen fermentation to improve feed digestibility and utilization. Plant secondary metabolites are natural substances with the potential ability to alter rumen fermentation without causing microbial resistance, and their residual effects can positively affect the animal end products (27, 28). Owing to the excellent antimicrobial activities of phytochemicals, they are considered as a potential modulator of the rumen microbiome to alter rumen physiology (29). Many experiments, including both *in vitro* and *in vivo* studies, have been conducted to explore the potential of phytochemicals on rumen fermentation to increase feed digestibility and reduce methanogenesis (30–32). Many of them have shown promising results, but applicability in terms of efficient animal production is questionable. Therefore, efforts are still underway to find an appropriate feed additive to mitigate rumen CH_4 production, simultaneously improving livestock production while reducing greenhouse effects on the environment. To accomplish this challenging task, an in-depth understanding of rumen development, microbial colonization, the interaction of rumen microbiome with the host, and diet is indispensable. Therefore, this review aims to provide insights into the effect of different phytogenic and dietary interventions on ruminal microbes to mediate rumen fermentation and methanogenesis to increase overall feed efficiency to make livestock production sustainable and more profitable.

ONTOGENESIS OF THE RUMEN AND INITIAL MICROBIAL COLONIZATION

The ruminant digestive system switches from monogastric to become fully active post-weaning rumen with the ability to digest fibrous feed. During the suckling period of the calf, milk bypasses the rumen due to the esophageal groove. Developed rumen comprises 60–80% of the total digestive system as compared to the monogastric stomach in early life. Besides this, rumen villi are not yet developed, which are necessary for the absorption of nutrients (33, 34). Rumen microbial populations exhibit an incredible impact on rumen structure and physiological development. Initial inoculation of rumen microbes in calves constitutes both aerobic and facultative anaerobic microbial taxa following birth, which later on mostly are replaced by anaerobic taxa (35). That is why 1-day-old calves have a massively different bacterial population as compared to 3-days-old calves (14).

The oxidative condition of the rumen is a primary regulator of shifts in the newborn rumen ecosystem, and redox has an inert impact on the colonization of methanogenic species (36). Ruminal bacteria such as cellulolytic species, *Ruminococcus flavefaciens*, and *Ruminococcus albus* and members of the *Prevotella* genus can already be detected on day one after birth. These microbes are involved in various rumen functions, such as cellulose and hemicellulose degradation (14). One of the primary changes observed throughout the rumen development includes modification in configuration within the *Bacteroidetes* phylum. In the developed rumen, this phylum is dominated by the genus *Prevotella* across several ruminant species (37). Nevertheless, during the primary stages of development, *Bacteroides* is the main genus within *Bacteroidetes* and is subsequently replaced by the *Prevotella* during the first 2 months (38).

Quick fluctuations in community configuration also affect methanogenic archaeal communities along with bacteria. Rumen methanogenic communities in calves and lambs have been detected as early as 20 min after birth. Like bacterial populations, the primary methanogenic population varies significantly between young and adult animals (39–41). Both preweaning calves and mature animals have a *Methanobacteriales* order, but rumen of preweaning calves contains two additional orders, *Methanosarcinales* and *Methanomicrobiales* (41). The compositional variations in rumen archaea lead to shifts in substrate utilization, methanogenic pathway, and extent of CH_4 production (42).

The establishment and colonization of microbiota play a key role in the development and function of the gastrointestinal tract (GIT), which is subsequently associated with higher body weight and feed efficiency of growing ruminants (43). Developing a rumen ecosystem during weaning age is key to getting improved growth rates and better health at a later stage of life (44, 45). The main objective of such strategies is to overcome the risk of undesirable health consequences associated with an altered gut microbiome in neonatal animals and restoration of the gut microbial community following dysbiosis. A complete understanding of early gut colonization is necessary to designing different effective strategies to manipulate the GIT microbiome. Although a wealth of literature is available on different aspects of rumen microbiome in adult animals and early colonization of gut microbiota, information regarding the role of host genetics and microbial interactions in the early development of the gut microbiota is limited.

MODULATION OF RUMEN MICROBIOME USING PHYTOGENIC FEED ADDITIVES

Ruminants can transform fibrous and non-fibrous plant material into valuable products like meat and milk with the help of rumen microbes (46). Rumen inhabits various microbes like bacteria, protozoa, fungi, archaea, and bacteriophages (47). A symbiotic relationship exists between rumen microbes and the host animal in which both provide coveted substance to each other mainly in three ways: (1) mastication and rumination expand the surface area of feed particles for microbial attachment and digestion, and consequently, microbes secrete fibrolytic enzymes for degradation of cellulose, and hemicelluloses;

(2) ruminal movements (peristalsis and antiperistalsis) bring microbes in contact with the fresh substrate by mixing of digesta and consequently yield fermentation products, especially VFA; and (3) elimination of fermentation products by belching and absorption is essential for keeping ideal conditions (pH) for microbial development and utilizing non-protein nitrogen (48).

Ruminal bacteria are the most prevailing microbiome, and their population measured by direct counts is usually 10^{11} cells per gram of rumen contents (4) comprising more than 200 species (49). Bacteria colonize inside rumen and have a major role in the metabolism of dietary carbohydrates and nitrogen and utilize fiber, starch, protein, and sugars. Generally, ruminal bacteria used homoserine lactone-based quorum sensing to communicate with each other (50). The most important genera of ruminal bacteria are *Butyrivibrio*, *Prevotella*, *Ruminococcus*, and *Pseudobutyrivibrio*. Mainly CH_4 emissions depend upon the abundance of H_2-producing bacteria in the rumen (51).

Ruminal fungi comprise 5–20% of the total microbiota in the rumen (52, 53). Anaerobic fungi are known as key players for the breakdown of lignocellulosic fiber (54). Anaerobic fungi are considered one of the most potent fiber-degrading agents, because of their active and extensive set of enzymes for the breakdown of plant polymers (55). Fungi produce enzymes vital for the digestion of plant materials, including cellulases, xylanases, mannanases, esterases, glucosidases, and glucanases (56). Rumen fungi also possess amylolytic (57) and proteolytic activities (58). The action of anaerobic fungi is promoted by the methanogenic archaea (59). However, the present understanding of rumen eukaryote function is far less than that of rumen bacteria, primarily due to the restricted annotation of the transcriptome and multiple-genome sequence availability (60). A recent *in vitro* study reported that a combination of anaerobic fungi (*Caecomyces*) and methanogens (*Methanobrevibacter*) have a greater ability to degrade lignocellulose and to produce CH_4 as compared to the combination of bacteria and methanogens, and whole rumen content enrichment (61).

Ruminal protozoa represent about 20–50% of total microbial biomass and are commonly grouped into flagellates and ciliates. The flagellate proportion to overall ruminal fermentation is negligible (62). However, ciliate protozoa have a fundamental function in rumen fermentation as they engulf fermentable carbohydrates (63) and prevent alternative bacterial fermentation that would otherwise decrease pH and increase the onset of lactic acid acidosis (64). There is a positive correlation between ruminal protozoa and volatile fatty acid and CH_4 production. Ciliate protozoa can enhance the metabolic output of the rumen microbiome; for instance, acetate, butyrate, iso-butyrate, and iso-valerate concentrations were improved in microcosms incubated with the protozoa population (65). The hydrogenosomes of rumen protozoa are involved in the production of H_2, which is subsequently converted to CH_4 by the methanogens through the hydrogenotrophic pathway (66, 67). Approximately 11% reduction in the CH_4 output has been observed due to the defaunation of protozoa (64, 68).

Archaea represent the third major domain of rumen microbes that constitute about 21% of the rumen microbiome (69). Methanogenic archaea belong to the phylum *Euryarchaeota* and are ubiquitously involved in methanogenesis (7, 69). Rumen methanogens have a synergistic association with bacteria and a symbiotic association with protozoa as <1% of the total microbial population (70). Different substrates are utilized during methanogenesis including formate, or acetate, methanol, H_2, methylamines, and CO_2 (71). Methane is produced mainly through three pathways: (i) primarily by reduction of CO_2 through the hydrogenotrophic pathway, (ii) less from the use of methyl groups (methylotrophic pathway), and (iii) even less through acetate (acetoclastic pathway) production (**Figure 1**). Methanogenic paths comprise three stages: exchange of the methyl set to coenzyme M (CoM-SH), reduction of methyl-coenzyme M with coenzyme B (CoB-SH), and reuse of heterodisulfide CoM-S-S-CoB (51, 68, 73).

The bacteriophage community is also an important component of the rumen microbial ecosystem. Studies have reported inconsistent findings of bacteriophage counts ranging from $>10^9$ particles of phages (74) to between 3×10^9 and 1.6×10^{10} particles per ml of rumen content (75). Bacteriophages possess a specific lysogenic ability against different bacteria that helps in bacterial mass turnover in the rumen. Due to a lack of information regarding the mechanisms of rumen phage–host interactions and the environmental factors affecting the relative proportions and dynamics of the phage population in the rumen, it is not possible to definitively determine whether the presence of phage in the rumen is disadvantageous or advantageous. However, possible functional consequences of rumen phages have been proposed as (1) the negative nutritional consequences of phage-induced bacterial lysis resulting in the recycle of nutrients within the rumen, (2) the positive effects of maintaining bacterial population diversity and facilitating gene transfer, and (3) the negative consequences of phage-mediated gene transfer.

Keeping in view their critical role in digestive physiology and nutrient metabolism, modulation of the rumen microbiome is envisioned as a practical strategy to mediate fermentation kinetics and methanogenesis. Modulation of the rumen microbiome can be possible through different dietary interventions; however, in this regard plant secondary metabolites possess a greater potential as compared with antibiotics to modulate the ruminal microbiome and mitigate CH_4 emission through diverse antimicrobial mechanisms such as perturbation of cell membrane, modulation of signal transduction or gene expression pathways, enzyme inhibition, and inhibition of bacterial colonization (76, 77). Plant secondary metabolites usually enhance the permeability and fluidity of the cellular membranes, further causing an efflux of metabolites and ions and ultimately leading to cell leakage and microbial death. Besides, they can also desirably manipulate the rumen metabolism by increasing the cell membrane's permeability of few specific rumen bacteria (78, 79). Putative mechanisms of actions mainly include disturbance of the cytoplasmic membrane, disruption of the proton motive force, electron flow, active transport mechanisms, and coagulation of cell composition (80).

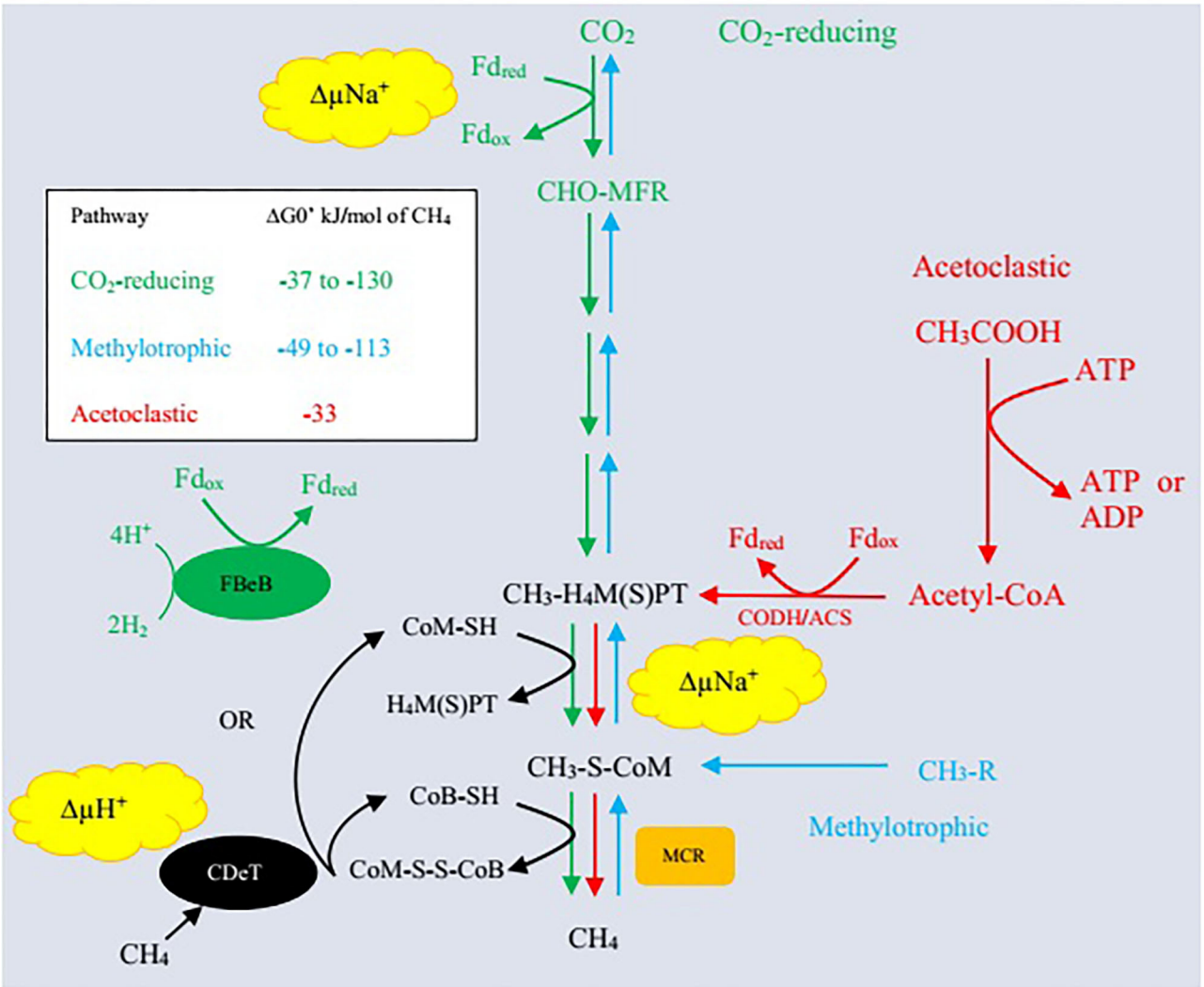

FIGURE 1 | Three enzymatic pathways of methanogenesis. Δ, CO_2-reducing pathway (hydrogenotrophic pathway); Δ, acetoclastic pathway; Δ, methylotrophic pathway; MFR, methanofuran; H_4MPT, tetrahydromethanopterin; H_4SPT, tetrahydrosarcinapterin; $\Delta G^{0'}$, standard free energy change; CH_3-R, methyl-containing compounds such as methanol, methanethiol, dimethylsulfide, monomethylamine, dimethylamine, trimethylamine, and tetramethylammonium; Fd_{red}, reduced form of ferredoxin; Fd_{ox}, oxidized form of ferredoxin; $\Delta\mu Na^+$, electrochemical sodium ion potential; $\Delta\mu H^+$, electrochemical proton potential; FBeB, flavin-based electron bifurcation; CDeT, cytochrome-dependent electron transfer; MCR, methyl-coenzyme M reductase; CODH/ACS, carbon monoxide dehydrogenase/acetylCoA synthase/decarbonlyase complex. Adapted from Lyu et al. (72).

GENETIC MANIPULATION OF RUMEN MICROBIOTA

The host diet has a major influence on the relative abundance and diversity of the rumen microbiome. However, genetic manipulation of rumen microbiota is also possible through different techniques as host genetics influences some heritable microbial traits (81–83). In this regard, recent biological techniques such as transgenesis are getting attention for improving the efficiency of animal production and reducing environmental impacts (84). New genome editing tools provide an efficient way to produce gene-edited ruminants, having resistance against certain diseases and specific product quality (85). In transgenic animals usually, a foreign gene of interest is inserted into its genome to express a desirable trait (86). In a study of pig transgenesis, neomycin phosphotransferase transgene has been evaluated using high-throughput sequencing. Neo-transgenic expression in transgenic pigs showed a significant increase in the relative abundance of some bacteria (*Firmicutes*, *Bacteroidetes*, and *Proteobacteria*) with a reduction of potentially harmful bacteria such as *Escherichia–Shigella–Hafnia* (87). A recent study by Yang et al. (88) showed inactivation of the ABO acetyl-galactosaminyl-transferase gene through a deletion of 2.3 Kb, potentially affecting the microbiota composition and its relative abundance (particularly *Christensenellaceae* and *Erysipelotrichaceae* families).

Presently, different studies are being focused to identify heritable microbes and microbial features in humans and animals, considering gut microbiomes as heritable phenotypes, (89). Elucidation of the association between host genetics and rumen microbiome composition will help to identify persistent microbial taxa and functions that are characteristic of efficient animals, thereby directly facilitating efforts to genetically select or permanently alter the rumen microbiome. Within the next 5–10 years, significant progress is expected regarding the relationship between gut microbiomes and the genetics of their ruminant hosts, unraveling an intricate network that paves the way for the genetic selection of heritable microbes and keystone microbial species. These efforts will rapidly advance microbial ecology research and animal production to efficiently and sustainably produce high-quality protein for human consumption to considerably contribute to global food security (90).

DIET–MICROBE INTERACTIONS

Emerging feeding techniques to limit CH_4 emissions are necessarily required both for preserving the environment and for increasing the efficacy of energy utilization. Different microbes are involved in the production of VFA (**Figure 2**). The rumen of dairy cow possesses diverse consortia of microbes that produce significant amounts of GHG gases (mainly CH_4) during feed digestion (48). Methodologies to divert rumen carbon and nitrogen metabolism away from these products offer opportunities for improving the efficiency of ruminant production by enhancing nutrient utilization while reducing GHG emissions.

Many studies have been conducted regarding the development of nutritional interventions to cut down CH_4 emissions from ruminants (91–93). With increasing food safety concerns, different natural compounds of plants are being considered ideal for moderating/mitigating CH_4 emissions (94). The diet fed to ruminants is the primary determinant of bacterial community structure (95–97). Co-oscillations of microbiota are very important to maintaining homeostasis in gastrointestinal ecology after dietary perturbations (98). It is important to adjust the animal diet according to their age and physiological condition and to provide proper time to adapt to different dietary changes. Diet is also considered as an important factor to ensure proper animal health and performance because digestion and utilization of nutrients mainly depend upon collaboration and competition of the microbiome. Important diet–microbe interactions are discussed as below.

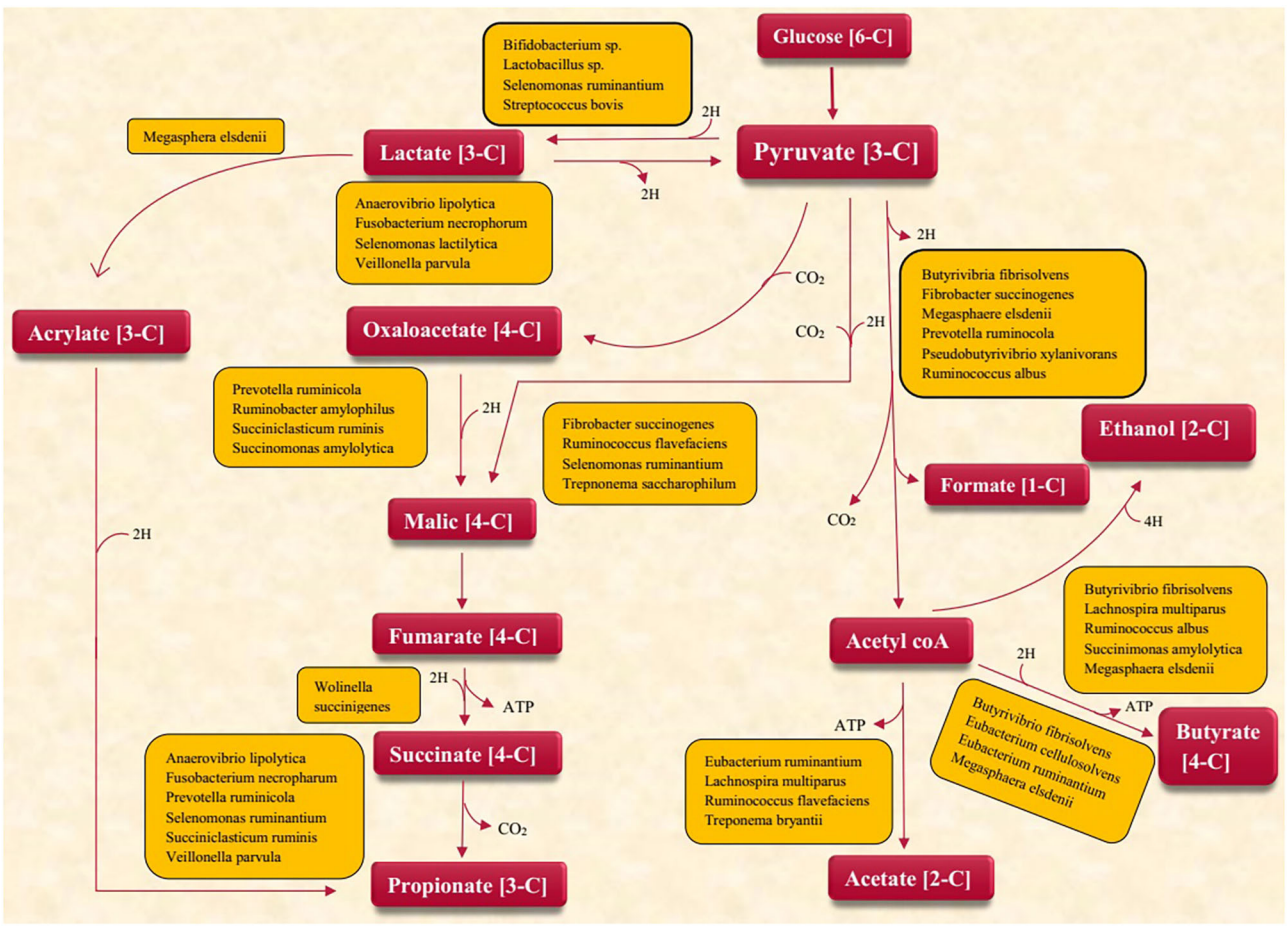

FIGURE 2 | Microbes involved in VFA production.

Carbohydrate–Microbe Interactions

Rumen microbiology involves the characterization of microbes and processes associated with fiber digestion (17, 99, 100). Rumen bacteria play a dominant role in fiber digestion, although anaerobic fungi and protozoa have been reported to contribute to lignocellulose breakdown by attacking lignocellulosic material differently (60, 64) and discharging different enzyme complexes (101). Microbial communities found in the rumen of cows fed total mix ration (TMR) vary as compared to pasture-fed cows, owing to the variable dietary composition (102, 103). Different rumen bacteria have shown associations with specific diets like *Fibrobacteraceae* with TMR and *Veillonellaceae* with pasture-based diets (104).

Feeding concentrate diets have shown to lower the ruminal pH, increase VFA concentrations and osmolality, and induce metabolic disorders (105). Moreover, high starch feeding substantially increases the activity of lactic acid consuming and producing bacteria in the rumen because these microbes are not susceptible to lower pH and hence opportunistically utilize higher substrate availability (106). In contrast, feeding a considerable amount of roughages may limit feed consumption, energy efficiency, and microbial protein synthesis in ruminants (107). Therefore, an increasing quantity of starch in the diet is considered as a promising strategy to decrease methanogenesis per unit of dry matter intake through shifting ruminal fermentation toward propionogenesis (108). It is mainly attributed to the fact that the supply of substrates and microbial growth depends upon the dietary fiber or starch contents (109). Increased dietary starch levels have shown a relatively lower proportion of cellulolytic bacteria *R. albus* and *R. flavefaciens*, owing to their higher sensitivity toward low pH. However, in an acidic ruminal environment *F. succinogenes* have shown to remain stable due to their gram-negative nature and different cell membranes than *R. albus* and *R. flavefaciens* (110). Feeding grain-based diets to cattle could reduce the bacterial diversity compared to forage-based diet (111). This reduction might be attributed to less availability of substrates for bacteria that ferment structural carbohydrates and the subsequent lower pH.

Feeding of high starch and low fiber diets has also been shown to enhance the growth of amylolytic bacteria in the rumen. Propionate is produced by *S. ruminantium* through decarboxylation of succinate (6). This bacterium is capable of using starch and sugar for its growth. Higher dietary starch contents have been shown to increase the concentrations of propionic acid in the rumen substantially. Furthermore *S. ruminantium* is also capable of using lactic acid to stabilize the rumen pH (112). Thus, an increase in the population of these bacteria can enhance the utilization of fermentable substrates generated in the rumen following a high concentrate diet. A facultative anaerobe (*S. bovis*) predominated in ruminants fed an increased amount of concentrate during lactic acidosis (109). The activity of *S. bovis* only increases as a result of low pH (<5.75) conditions in the bovine rumen (113). According to McCaughern et al. (114), feeding a high-starch (220 g/kg of DM) diet to dairy cows could reduce rumen pH (0.15 units lower than normal pH) and increase milk yield (0.09 kg/d) and milk protein content (2.8 g/kg). Feeding a high-starch diet can directly affect the colonic lumen environment, which in turn alters the lumen-specific functional taxonomic groups (*Akkermansia*, unclassified *Christensenellaceae*, and *vadinBB60*). Consequently, the colonic epithelium makes a new niche that triggers cell apoptosis to achieve a functional transformation (98). These studies suggested that microbe–host interaction is vital for remodeling of hindgut homeostasis to allow adaptation to dietary perturbations.

Silage from various sources harbors different rumen microbial communities. Feeding of alfalfa silage increased the relative abundance of *F. succinogenes* and *R. flavefaciens* while reducing CH_4 production in the cow rumen as compared to sweet sorghum silage. However, populations of *Ruminococcus albus* and *Ruminobacter amylophilus* showed no change (115). Contrarily, sheep fed an alfalfa hay diet had higher ruminal *Fibrobacter succinogenes* compared to *Ruminococcus* (116). According to Guo et al. (117), fermented corn stover showed a positive effect on ruminal bacterial diversity favoring four bacterial phyla; *Bacteroidetes*, *Lentisphaerae*, *Firmicutes*, and *Fibrobacteres*, which constituted 77% of total bacterial abundance. Additionally, feeding of fermented corn stover shifted the rumen fermentation kinetics in cows through increasing the relative abundance of *Prevotella* and stabilizing the rumen microbial ecosystem. Recently, it has been reported that feeding temperate grasses produce less enteric CH_4 than tropical grasses in ruminants (118) as feeding low-quality tropical grasses emitted 17 g CH_4/kg DM intake. However, a 10–25% decrease in CH_4 production has been observed, when foliage and pods of trees and shrubs are included in the cattle diet (118).

A recent meta-analysis showed that feeding high-forage diets (>40% DM) reduced milk production (0.087 L/d) and milk lactose content (0.065 g/100 g) compared to high-concentrate diets (>40% DM) in sheep. However, fat content and conjugated linoleic acid concentrations were higher in the high-forage group (119). These findings convincingly reveal that the appropriate ratio of roughage and concentrate is required to optimize the rumen microbial ecosystem for better digestion and utilization of the dietary components while minimizing CH_4 emission. Chemostatic feedback regulation (energy feedback), physical fill and feed passage rate of concentrate, and forage-type diets are involved in affecting the DM intake of ruminants. Moreover, inoculation of silage with specific groups of beneficial microbes can positively influence rumen fermentation kinetics and performance of animals on a sustainable basis.

Interaction of Dietary Fat With Rumen Microbiome

Generally, supplementation of fatty acids (FA) is not required for microbial proliferation in the rumen because microbes can synthesize their own FA. Basal feed ingredients, including forages and grains, provide about 3–3.5% fat on a dry matter (DM) basis. However, for high-producing dairy cows, additional fat supplementation up to 2% of rumen-active fat (vegetable blends, oilseeds) and rumen-inert fat is usually recommended to make total dietary lipids up to 6–7% of DM (120). These dietary lipids are usually enriched in polyunsaturated fatty acids (PUFA) (121), which can make complexes with bacterial cell walls and are

considered toxic to gram-positive bacteria in the rumen (122). In most ruminant diets, fat is below 5% of total DM. Higher dietary fat contents, primarily unsaturated FA, are discouraged owing to their adverse impacts on ruminal bacteria and feed degradation (123). However, rumen microbiota can detoxify unsaturated FA through the biohydrogenation process to reduce/eliminate the adverse effects on rumen fermentation (121, 124).

The addition of fats from plant or animal sources is an accepted approach for mitigation of CH_4. However, consideration of fat supplementation to mitigate enteric CH_4 emission depends on the cost and expected adverse effect on feed intake and digestibility (19). Improving the nutritive quality (high fat and fiber digestibility) of the offered diets has shown to reduce the DM intake in lambs but showed no effect on total tract digestibility of DM, organic matter (OM), crude protein, acid detergent fiber (ADF), and neutral detergent fiber (NDF) contents (125). Adding fat up to 6–7% of DM has shown no adverse effects on total tract digestibility (126). Recent studies have reported no effects of rumen-protected fats on NH_3-N concentrations, total VFA, and overall bacterial population in sheep (127). Changes in rumen microbiota (*Acetitomaculum*, *Lachnospira*, and *Prevotella*) caused by an increased proportion of concentrates were considerably more significant than fat (128). *Fibrobacter* and *Ruminococcus* were most adversely affected among different bacterial genera, but such effects were highly variable for *Butyrivibrio* and *Prevotella*. These two genera (*Butyrivibrio* and *Prevotella*) include many species with diverse functions in metabolic pathways (129).

A decrease or no change was observed in major protozoa genera in response to the addition of linseed oil, particularly in high-concentrate diets (130). Moreover, increasing the degree of unsaturation reduces the protozoal count, but due to high random and animal variations, this change can be challenging to assess, which may explain inconsistent experimental data (131). Dietary supplementation of camelina oil has not shown any effect on ruminal protozoa (132), but a decrease in bacterial N and the number of cellulolytic bacteria was observed in diets supplemented with 8% dietary lipids (133). However, Bayat et al. (134) reported that the inclusion of camelina oil in the diet exhibited no effects on the relative abundance of protozoa, total bacteria, methanogens, fungi, and fiber-degrading bacteria.

Lipids from oilseeds, vegetable oils, and rumen-protected fat of vegetable oils are usually used as energy sources for dairy cattle (135). Oilseeds can be one of the efficient ways to reduce enteric CH_4 production to mitigate CH_4 emission from ruminants. Plant oils can mitigate CH_4 by directly inhibiting rumen protozoa and methanogens and increasing the biohydrogenation of PUFA to act as a sink for hydrogen produced by rumen microbes (136). The utility of lipids to reduce enteric CH_4 production is a better strategy as compared to antibiotics and ionophores like monensin. Several studies have reported adverse effects of FA, especially PUFA, on methanogenesis in the rumen (136). The anti-methanogenic effects of PUFA generally get intensified with the increase in double bond number per FA, as suggested by Czerkawski and Clapperton (137).

Supplementation of fat has been shown to reduce CH_4 emission in ruminants consistently. However, various factors such as fat source, FA profile, basal diet, and fat type can affect the anti-methanogenic efficiency of dietary fats (93). This reduction in CH_4 emission by dietary fat is mainly through the depressed fiber digestion in the rumen (138). However, according to McGeough et al. (125), a highly digestible fiber diet with higher fat content tended to increase CH_4 emissions per kg of DMI and OMI, while the same amount of fat showed no effect on these parameters in a diet with low fiber digestibility. The inclusion of fat in a high-concentrate diet of sheep improved fat and conjugated linoleic acid contents of milk (119). Bayat et al. (139) reported a decrease in daily CH_4 emission in lactating cows fed a low concentrate diet supplemented with sunflower oil.

Recently, moringa and camelina oils have shown to effectively reduce enteric *in vitro* CH_4 production in different TMR through modulation of rumen microbes and shifting rumen kinetics (140, 140). Variable effects of vegetable oils and unsaturated FA on CH_4 emission might be associated with the double bond number per FA, type of oil (free oil or whole seed), and composition (roughage-to-concentrate ratio) of the rations (134). An *in vitro* study of Vargas et al. (141) shows that supplementation of vegetable oil (sunflower and linseed) at 6% in high-concentrate TMR has the potential to reduce CH_4 emission (up to 21–28%), butyrate concentration, and A:P, while increasing propionate concentration. Recently, a meta-analysis showed that addition of nitrates and vegetable oils in cattle diet has the ability to reduce CH_4 emission up to 6–20% (118). Considering different studies regarding the reduction of CH_4 emission, the addition of plant oils to ruminant rations is suggested as a feasible nutritional strategy with a cleaner repercussion on the environment.

DIETARY MANIPULATION OF RUMEN FUNCTION USING NATURAL FEED ADDITIVES

Dietary changes have been reported as a major factor that influences the dynamics of rumen microbial populations and resultant metabolic shifts leading to significant changes in ruminant production (142, 143). Many dietary interventions have been used in ruminants for the manipulation of the rumen microbiome to improve overall feed efficiency while reducing methanogenesis. A few dietary methodologies have been assessed for enhancing rumen fermentation, mainly to reduce CH_4 emission. These strategies were focused to (i) improve feed efficiency using the quality feed, (ii) shift rumen fermentation pathways using assorted feed additives, and (iii) genetically manipulate host animals using selective breeding. Each strategy has some potential advantages and limitations. Recent issues of drug residues and antibiotic resistance have shifted the interest toward natural feed additives with potential abilities to modulate performance in ruminants. Many encouraging results have been observed by the application of different feed additives, including organic acids, probiotics, enzymes, and phytochemicals. Ideally, feed additives should diminish CH_4 emission, enhance animals' energetic efficiency by increasing propionate concentration,

improve N_2 utilization efficiency by decreasing its excretion, optimize rumen pH, and improve fiber digestion (144).

Most important natural feed additives are phytochemicals produced by plants as secondary metabolites with diverse biological activities. Some potential effects of these feed additives and their mechanism of action as rumen modulators are described as below.

Plant Secondary Metabolites as Rumen Modulator

Despite the sustainability of functional redundancy in the rumen microbiome, plant secondary metabolites have shown significant manipulation of the rumen microflora leading to a shift in fermentation dynamics and milk production in lactating animals (29, 31, 145–147). They have also shown to reduce the methanogenesis both *in vitro* and *in vivo* (29, 31, 76, 148, 149). Recently, numerous plant extracts have been investigated for their capacity to manipulate gut physiology and antimicrobial activity. Some plant metabolites, for example, saponins, tannins, and essential oils (EO), have shown promising potential for decreasing CH_4 emission from animals. They have shown significant impacts on methanogens as well as protozoa, feed degradation/absorption, and fermentation parameters.

Effect of Saponins on Rumen Methanogenesis and Fermentation Characteristics

Saponins are a class of plant secondary compounds with diverse chemical compositions and biological activities (31). Saponins comprise mainly sapogenins and glycosides found mostly in angiosperms. Steroidal and triterpenoid saponins are two significant groups of saponins, which protect plants from bacterial and fungal invasions (150). Saponin-rich plants such as lucerne and soybeans are broadly utilized for ruminant feeding. Additionally, *Quillaja saponaria* (soapbark), *Yucca shidigera* (yucca), and *Sapindus s*p. (soap berries) are considered as well-known sources of saponins (151). Saponins have a fat-soluble nucleus, and they have shown antibacterial, antitumor, and anti-inflammatory properties in animals (152, 153). Saponins mediate rumen fermentation mainly by decreasing protein degradation and concentrations of urea and NH_3 in the rumen, leading to an increased flow of amino acids to the small intestine. Potential effects of saponins are associated with N_2 metabolism, mainly through their lethal effect on protozoa, which are primarily responsible for proteolytic activity in the rumen (154). Saponins reduce the protozoal population and some methanogens associated with protozoa, although their effect on methanogens does not always correlate with the effect on protozoa (31). The interaction of sterol moiety with saponin, present in the protozoa membrane, has an association with the antiprotozoal effect of saponins (154).

It is generally expected that a reduction in the population of methanogens can decrease CH_4 emissions. Extracts of *S. sesban* have shown to decrease protozoal and methanogen populations but surprisingly did not decrease CH_4 production (155). There was a frail relationship between methanogenesis and methanogens in the rumen. This is mainly because the enhanced expression of some methanogenic genes may lead to enhanced methanogenesis that ultimately compensated a decrease in the overall number of methanogens (156). These findings provide explicit insight regarding, managing gene interactions from the microbiome to enhance nutrient utilization efficiency.

Tea saponins have been extensively used in many studies *in vitro* and *in vivo* to evaluate the effects on rumen fermentation and methanogenesis. Variable results regarding microbial population and rumen fermentation parameters have been observed in response to the supplementation of different sources of saponins (**Tables 1**, **2**). Despite the decline observed in methanogenesis through a direct decrease in protozoa

TABLE 1 | Effect of saponins on rumen microbial population.

Sources	Test system/dose	Diet	Total bacteria	Protozoa	Methanogens	*F.S*	*R.F*	*R.A*	*B.F*	References
Tea saponin	*In vivo* or *in vitro* both in ewe 3 g/d	TMR + wildrye hay	=	↓	=	↑	=	=	=	(157)
Tea saponin (Lerak)	*In vitro* rumen fluid from	Cassava leaf silage	=	=	=	=	=	=	NF	(158)
Tea saponin (Hibiscus)	Cattle (2 and 4%)		=	=	=	=	=	=	NF	
Quillaja saponin	*In vitro* rumen cows (0.6 g/L)	TMR	↑	NF	=	NF	NF	NF	NF	(159)
Combination (Enterolobium cyclocarpum and Gliricidia sepium)	*In vivo* or *in vitro* both in heifer	TMR 3.3% of 15% DM	=	=	=	NF	NF	NF	NF	(160)
Tea saponin	*In vitro* rumen fluid from cows 0.77%	F:C (50:50)	=	=	=	NF	NF	NF	NF	(161)
Tea saponin	Chambers 0.52% *in vitro* bottles	TMR	NF	↓	NF	NF	NF	NF	NF	(162)
Quillaja saponin	Open chambers (0.6 g/L)	F:C (50:50)	=	↓	↓	=	↑	↑	NF	(163)

F.S, Fibrobacter succinogenes; R.F, Ruminococcus flavefaciens; R.A, Ruminococcus albus; B.F, Butyrivibrio fibrisolvens; NF, not found; ↑, increase; ↓, decrease; =, no effect.

TABLE 2 | Effects of saponin on methanogenesis, rumen fermentation, and feed degradability.

Sources	Test system/dose	Diet	CH_4	NH_3	tVFA	DMI	Acetate	Butyrate	Isobutyrate	Propionate	Isovalerate	Valerate	Acetate/ Propionate	DMD	References
Tea saponin	*In vivo* or *in vitro* both in ewe 3 g/d	TMR + wildrye hay	=	↓	↑	=	=	↑	↑	↑	↑	=	↓	↑	(157)
Tea saponin (Lerak)	*In vitro* rumen fluid from cattle (2 and 4%)	Cassava leaf silage	=	↓	=	NF	↑	↑	=	↑	=	↑	NF	↑	(158)
Tea saponin (Hibiscus)			↑	↓	=	NF	↑	=	=	↓	=	=	NF	↑	
Quillaja saponin	*In vitro* rumen fluid from cows (0.6 g/L)	TMR	=	↓	=	NF	=	=	=	=	=	=	=	=	(159)
Combination of kulthi, patha, and aritha	*In vitro* rumen fluid from male buffaloes 2%	F:C (80:20)	↓	NF	↓	NF	=	↓	=	=	=	=	↓	↑	(164)
Combination of Enterolobium cyclocarpum and Gliricidia sepium	*In vivo* or *in vitro* both in heifer	TMR 3.3% of 15% DM	=	NF	=	=	=	=	=	=	NF	NF	=	=	(160)
Sapindus mukorossi fruits acetone extract	Buffalo rumen 125 ml bottles fitted 0.5 ml	Oat hay	↓	=	NF		=	=	=	NF	NF	NF	=	=	(165)
Alfalfa saponins	*In vivo* lamb 0.4%	F:C (50:50)	NF	NF	NF	=	NF	NF	NF	NF	NF	NF	NF	↑	(166)
Tea saponin	Open chambers 0.52% *in vitro* bottles	TMR	↑	=	=	↓	=	=	=	NF	NF	NF	=	=	(162)

F:C, forage to concentrate ratio; TMR, total mixed ration; tVFA, total volatile fatty acid; DMI, dry matter intake; DMD, dry matter degradability; NF, not found; ↑, increase; ↓, decrease; =, no effect.

populations under *in vitro* conditions, the same plant extract (0.52% on DM basis) failed to reduce daily CH_4 production in lactating dairy cows (162). This suggests that the impacts of tea saponins under *in vitro* conditions must be confirmed *in vivo* to develop effective CH_4-mitigating strategies.

Inactivation of saponins has been observed through deglycosylation into sapogenins by the rumen microorganisms that lead to the transitory antiprotozoal property of saponins. There are two approaches to improve the effectiveness of saponins and reduce their degradation by rumen microbes. One possible method is to use a combination of saponins with glycosidase-inhibiting iminosugars (167). The second option is altering the saponin structure, such as by combining ivy saponins with stevia extract. Hederagenin bis-succinate (HBS) obtained by hydrolysis of ivy fruit extract has shown to shift fermentation toward propionate, attributed to its structural modifications that mediated the diversity of bacterial communities (167).

Recently, tea saponins have been supplemented in alfalfa hay and soybean hull-based fiber diets and exhibited their ability to alter ruminal lipid metabolism in cattle through reducing the relative abundance of *Lachnospiraceae* (168). Furthermore, tea saponins have also been shown to effectively decrease N_2 emission in sheep (157). However, studies have revealed that the activity of saponins fluctuates and even reduces during long-term studies (158), probably because of microbial adaptation (169). Moreover, saponins can increase the propionate ratio at the expense of both acetate and butyrate (158). Studies have shown that a combination of garlic oil, nitrate, and saponins can additively lower CH_4 emission with similar rumen fermentation and degradability (163). Archaeal growth was inhibited by all treatments, but the abundance of *F. succinogenes R. albus*, and *R. flavefaciens* varied. According to Liu et al. (157), tea saponin did not affect methanogens and the total bacterial population including *R. flavefaciens R. albus*, and *Butyrivibrio*. However, protozoa were effectively reduced in response to tea saponins.

All saponins have no inherent antiprotozoal activity; that is why their biological activity can be affected by even small changes in their structure. For instance, sapogenins like asiatic acid and madecassic acid have more ability for the inhibition of protozoa than their corresponding saponins (Re and Rh_1 and madecassoside). Therefore, further research is warranted to understand the deglycosylation of saponins and the nature of their antiprotozoal activity to devise effective ways to use saponins for CH_4 mitigation in ruminants (170).

Effect of Tannins on Rumen Methanogenesis and Fermentation Characteristics

Tannins are polyphenolic compounds with molecular weights ranging from 500 to 5,000 Da with two major groups [i.e., condensed tannins (CT) and hydrolyzable tannins (HT)]. Tannins can bind with dietary proteins, starch, and sugar by making strong complexes at pH 3.5–7 (150). Tannins are widely distributed in different plant species, particularly in cereals, legumes, and fruits. They can limit the digestibility and nutritional value of plants considerably when their concentration reaches more than 5% (171). The action of tannins in the rumen is not entirely well-conceived yet (172). Although they possess bacteriostatic effects, the association of tannins with the rumen microbes is different, as hydrolyzable tannin is more susceptible to microbial hydrolysis than condensed tannin (173). They can limit the degree of microbial hydrolysis along with direct inhibition of methanogens. Additionally, they can also lower methanogenesis indirectly by decreasing H_2 availability by reducing fiber digestion (Figure 3). Tannins can modify the ruminal microbiome, reduce

TABLE 3 | Effect of tannins on rumen microbial population.

Sources	Test system/dose	Diet	Total bacteria	Protozoa	Methanogens	*PV*	*RC*	*RB*	*BV*	References
Acacia mearnsii	*In vivo* lamb 15 g daily dose	TMR	NF	NF	NF	↓	=	NF	NF	(176)
Chestnut tannin extract	*In vitro* Ewe rumen 16 g/kg DM of CHT extract	TMR	=	=	=	=	=	=	=	(177)
Tannic acid	*In vivo* cattle 16.9 g TA/kg DM	(TMR) Low CP	=	NF	NF	=	=	NF	=	(178)
		High CP	=			=	=		=	
DFPP condensed tannin (6.9%)	*In vitro* steers DFPP levels 1% 2% 3% 4%	F:C (30:70)	NF	↓ ↓ ↓ ↓	NF	NF	NF	NF	NF	(179)
HT Chestnut Tannic acid Gallic acid	Beef cattle 2% 1.5% 1.5%	Alfalfa silage	NF	= = =	NF	NF	NF	NF	NF	(180)
Tannin extracted from pomegranate peel	Lambs 29% 25% 30%	Recycled poultry bedding	= ↓ ↓	NF	NF	NF	↓ ↓ ↓	NF	NF	(181)
HT Gallic acid	*In vitro* 0.5% 1% 2%	TMR	NF	= = =	NF	NF	NF	NF	NF	(182)
HT Syzygium cumini	*In vivo* Lambs 14.08 and 4.29 g/kg DM	F:C (50:50)	=	↓	NF	NF	NF	NF	NF	(183)
CT Machilus bombycina			↑	=						
Tannin from chestnut, valonea, sumac and grape seed	*In vitro* Non-lactating cows 1.5 g/d	TMR	NF	NF	=	NF	↓	NF	NF	(184)
i) HT chestnut, tara	*In vivo* lamb 40 g/kg commercial extract	Concentrate	= =	= ↓	= =	↓ =	= =	= =	↓ =	(185)
ii) CT mimosa, gambier			= =	= ↓	↓ ↓	= ↓	= =	↓ =	= ↓	
HT chestnut	*In vivo* sheep 10%	Grass hay	NF	NF	NF	= =	↑	= =	↑	(186)
CT mimosa							↓		↓	

PV, Prevotella; RC, Ruminococcus; RB, Ruminobacter; BV, Butyrivibrio; NF, not found; ↑, increase; ↓, decrease; =, no effect.

protein degradation, decrease methanogenesis, and inhibit FA biohydrogenation (174, 175).

Variable results regarding the shifting of microbial population and rumen fermentation parameters have been observed in response to the supplementation of different sources of tannins (**Tables 3**, **4**). Studies have reported quite different effects of tannin supplementations regarding CH_4 mitigation. Some studies have also shown that tannins indirectly impede the degradation of fiber (76). A recent study showed that supplementation of acacia tannin (15 g/d/animal) reduced short-chain fatty acids (SCFA) and acetate (molar percentage) in lambs affected by gastrointestinal nematode infection.

TABLE 4 | Effects of tannins on methanogenesis, rumen fermentation, and feed degradability.

Sources	Test system/ Dose	Diet	CH_4	NH_3	tVFA	DMI	Acetate	Butyrate	Isobutyrate	Propionate	Isovalerate	Valerate	Acetate/ Propionate	DMD	References
Tannin-containing hay	*In vivo* cows and heifers	Hay	↓	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	(187)
ATE	*In vivo lambs* 42 g/kg DM	Urea-containing diet	=	↓	=	=	↓	=	NF	↑	NF	↑	↓	↓	(188)
Acacia mearnsii	*In vivo* lamb 15 g daily dose	TMR	NF	=	NF	NF	↓	↑	=	=	NF	↑	=	NF	(176)
lipid encapsulated-ATE	*In vitro* 24 h	TMR	↓	↓	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	(189)
Oak tannin extract	*In vivo* lactating cows 169 g/DM	TMR including linseed	=	NF	NF	=	NF	NF	NF	NF	NF	NF	NF	=	(190)
DFPP condensed tannin (6.9%)	*In vitro* steers DFPP levels	F:C (30:70)													(179)
	1%		↓	=	=		↓	=		↑			↓	↑	
	2%		↓	=	=		↓	=		↑			↓	↑	
	3%		↓	=	=		↓	=	NF	↑	NF	NF	↓	↑	
	4%		↓	=	=		↓	=		↑			↓	↑	
i) HT chestnut, tara ii) CT mimosa, gambier	*In vivo* lamb 40 g/kg commercial extract	Commercial concentrate diet	NF	=	=	NF	=	=	↓	=	↓	=	=	NF	(185)
				=	=		=	=	=	=	=	=	=		
				=	=		=	=	=	=	=	=	=		
				=	=		=	↓	=	=	=	=	=		
Quebracho tannin extract	Crossbred heifers,	Low-quality tropical													(191)
	1%	*Pennisetum*	=	=	=	=	=	=	=	=	=	=	=	=	
	2%	*purpureum* grass	=	=	=	=	=	=	=	=	=	=	=	=	
	3%		↓	=	=	=	=	=	=	↑	=	=	↓	=	
	4%		↓	=	=	↓	=	=	=	↑	↓	=	↓	↓	
40% distillers grains and solubles with CT	Cannulated crossbred beef heifers 2.5% CT extract	High protein finishing diets	↓	=	↓	=	=	↑	=	↓	=	=	↑	↓	(192)
Tannic acid	*In vivo* cattle	(TMR)	NF												(178)
	16.9g TA/kg	Low CP		↓	↓	=	↑	=	=	=	↓	↓	↑	↓	
		High CP		↓	↓	=	↑	=	=	=	↓	↓	↑	↓	
HT	Beef cattle	Alfalfa silage													(180)
Chestnut	2%		=	=	↑	=	=	=	↑	=	=	=	=	=	
tannic acid	1.5%		=	↓	=	=	=	=	↑	=	=	=	=	=	
gallic acid	1.5%		↓	=	↑	=	=	=	↑	=	=	=	=	=	
HT syzygium cumini CT Machilus bombycina	*In vivo* lambs 14.08 and 4.29 g/kg DM	F:C (50:50)	↓	↑	↓	=	↓	↓	=	↓	NF	↓	↓		(183)
			↓	↓	↓	=	↓	=	=	↓		↓	↓		

(Continued)

TABLE 4 | Continued

Sources	Test system/Dose	Diet	CH_4	NH_3	tVFA	DMI	Acetate	Butyrate	Isobutyrate	Propionate	Isovalerate	Valerate	Acetate/ Propionate	DMD	References
HT	*In vitro*	TMR												NF	(182)
gallic acid	0.5%		=	=	=	=	=	=	=	=	↓	=	=		
	1%		=	=	=	=	=	=	=	=	↓	=	=		
	2%		=	=	=	=	=	=	=	=	↓	=	=		
CT	*In situ* ram CT levels	Lucerne silage	NF		NF	NF	NF	NF	NF	NF	NF	NF	NF		(193)
Cistus ladanifer	4%			↓										↓	
	8%			↓										↓	
	12%			↓										↓	
Tannin extracted from pomegranate peel	Lambs 29%	Recycled poultry bedding	NF						NF					NF	(181)
	25%			=	=		=	=		=	=	=	=		
	30%			↓	=		=	=		=	=	=	=		
				↓											
Combination of TA and AF	*In vitro* TA (0.02 g) + AF (0.02 g) + Wheat barn (0.01 g)	Commercial concentrate diet	↓	NF	=	NF	=	NF	NF	=	NF	NF	NF	=	(194)
CT	*In-vitro* incubation	Cassava silage	NF		NF				NF		NF	NF	NF		(195)
	2.5%			=		↑	↑	=		=				=	
	5%			=		↑	↑	=		=				=	
	7.5%			=		↑	↑	=		=				=	
HT	*In vitro* Sheep	Acacia nilotica		NF										NF	(196)
Acacia nilotica	25%	leaves	↑		=	NF	↑	↓	↑	↑	↑	↓	↑		
	50%		↓		↓		↑	↓	↓	↓	↑	↓	↑		
	75%		↓		↓		↑	↓	↓	↓	↓	↑	↑		
	100%		↓		↓		↑	↓	↓	↓	↓	↑	↑		
Chestnut tannin, glycerol	*In vitro* Bull Chestnut, 30% Glycerol, 30% Both 60%	Ensiled cassava leaves F:C (60:40)				NF									(197)
			↑	↓	=		=	=	=	=	↑	=	=	=	
			↓	↑	=		=	↑	↑	=	=	=	=	=	
			=	↓	=		=	=	=	=	↑	=	=	=	
HT	*In vivo* sheep	Oil diets	NF	NF										NF	(186)
Chestnut	10%														
CT					↑	↑	=	=	↑	=	↑	=	↑		
mimosa					↓	↓	=	=	↓	=	↓	=	↓		

F:C, forage to concentrate ratio; TMR, total mixed ration; tVFA, total volatile fatty acid; DMI, dry matter intake; DMD, dry matter degradability; NF, not found; ↑, increase; ↓, decrease; =, no effect; HT, hydrolysable tannins; CT, hydrolysable tannins; DFPP, dragon fruit peel powder; TA, tannic acid; AF, Allium fistulosum L.; ATE, acacia tannin extract.

Furthermore, supplementation also increased the diversity and abundance of butyrate-producing and other beneficial bacteria (including probiotic species like *Bifidobacterium* and *Lactobacillusamino*), while enhancing the amino acid metabolic pathways and purine, pyrimidine, and sphingolipid metabolism (176). However, the precise mechanism of action and extent of contributory effects of acacia tannin on the ruminal microbiome are still not clear. Supplementation of tannic acid has shown to reduce the CP digestibility and CH_4 production in beef cattle (198). Dietary supplementation of HT (chestnut) and CT (quebracho) increased the relative abundance of *Butyrivibrio fibrisolvens* by 3 and 5-fold, respectively, in the rumen of dairy sheep. On the other hand, they decreased the *B. proteoclasticus* population by 5 and 15-fold, respectively (199). Inhibition of rumen bacteria by CT is probably due to interactions between CT present in the tannin structure and the specific substrate (e.g., protein, bacterial cell walls, etc.) to which it binds (200). The addition of CT extracts to the diet reduced populations of methanogenic archaea and some cellulolytic bacteria (*R. flavefaciens*) (201). These reports suggest that dietary sources of HT and CT can affect the rumen microbiome quite differently owing to their structural variations.

Different sources of tannin have been used to mitigate CH_4 emission while increasing animal performance. Gallic acid is a phenolic monomer and one of the ruminal decomposed metabolites of tannic acid (173), and it serves as an essential bioactive component in modifying the rumen fermentation. As a subunit of HT, gallic acid has the potential to decrease the environmental impact of ruminants (by lowering CH_4 and NH_3 emissions) without decreasing animal performance (180). Gallic acid (0.015%) decreased the urine nitrogen emissions by 28.5% (CP 0.11% DM) and 30.9% (CP 0.15% DM) when applied to the soil (202). A recent study showed that gallic acid can inhibit undesirable microorganisms such as *Clostridium*, *Listeria*, and *Escherichia coli* during ensiling and can improve fermentation quality and protein preservation (203). Extracts of HT (tara) and CT (mimosa and gambier) inhibited the activity of methanogens and protozoa without affecting ruminal fermentation and animal production (185). Condensed tannins have shown better protein efficiency and growth rate of lambs as it can protect dietary proteins (e.g., soybean meal) from ruminal degradation, leading to reduction in digestive losses (204). Recently an *in vitro* study of Saminathan et al. (205) showed that tropical legumes having CT with different molecular weights can serve as potential feed additives to mitigate CH_4 production with no adverse effects on rumen fungal microflora and fiber digestion. Contrarily, Rira et al. (196) reported that *in vitro* HT (*A. nilotica*) are more promising for suppressing methanogenesis than CT (from *C. calothyrsus* and *L. leucocephala*). Chestnut tannin possesses sufficient potential to reduce methanogenesis, without compromising feed efficiency and animal performance due to its neutral effect on NDF digestibility (177). According to Witzig et al. (184), CH_4 emission was reduced in response to monensin and chestnut tannin supplementation, owing to the lower abundances of *M. ruminantium* and *M. stadtmanae.*

Tannic acid (0–1.25 mg/mL) can alter microbial activities and improve feed efficiency in ruminants. However, the increased tannic acid concentration may lead to the complete inhibition of ruminal bacteria in sheep (206). Mimosa CT could reduce the abundance of specialized fibrolytic bacteria and inhibit the biohydrogenation process as compared to chestnut HT. Further investigations are required to evaluate the impact of different sources of tannins on ruminal biohydrogenation (186). Hydrolyzable tannins are considered more suitable for CH_4 mitigation than CT. *A. nilotica* (HT) showed a more potent inhibitory effect on CH_4 production compared to *C. calothyrsus* and *L. leucocephala* (CT). It may be attributed to the fact that HT (e.g., gallic acid subunits) directly inhibit methanogens, but the action of CT on rumen CH_4 production is variable (172, 207). However, long-term trials are required to assess the possible adaptation of rumen microbes toward the optimal level of HT and its subunit, gallic acid, to avoid their adverse effects on animal performance (196).

Tannin containing hay has been shown to reduce the CH_4 emission (5.4 DM vs. 3.5 ml/g) and urea N excretion in beef cattle (187). To reduce the adverse effect of tannin on DM intake, the encapsulation of tannin extract could be considered as a better strategy as the slow release of tannin also improves its utilization (208). Recently, Adejoro et al. (189) determined the effect of crude (40 g/kg feed) and lipid encapsulated-acacia tannin (50 g/kg feed) extracts on sheep fed TMR. They reported a 30% and 19% reduction in CH_4 production (g/kg DM) with crude and encapsulated-acacia tannin, respectively. However, crude tannin also imparted an adverse effect on NDF digestibility compared to encapsulated-acacia tannin. Supplementation of tannin could reduce the NH_3 toxification which is usually produced in response to NPN addition in ruminant diets (208). More recently, Adejoro et al. (188) reported that supplementation of 42 g acacia tannin /kg feed DM did not reduce CH_4 production in lamb fed nitrate or urea as an NPN source. A possible reason is the comparatively higher affinity of acacia tannin for feed protein than microbial protein or microbial enzymes (209). Tannin has the potential to reduce the excretion of a more volatile form of N into the environment by decreasing rumen degradability of CP and shifting N excretion from urine to feces (210). A meta-analysis showed reduction in ruminal ammonia N (16%), milk urea (9%), and urinary N excretion (11%) in response to supplementation of tannin in lactating dairy cows. However, tannin exhibited no effect on fat- and protein-corrected milk yield (211). A short-term effect of *A. mearnsii* (30 g/kg) showed a negative effect on CH_4 production in dairy cows.

Dietary supplementation of oak tannin has also been shown to reduce the urinary N excretion by 12% while increasing α-linolenic acid content in milk by 17.7% without affecting CH_4 production (190). Such divergent findings may possibly be due to different dietary concentrations of tannin and a variable number of hydroxyl groups in their structure (207). Studies have suggested an association of milk FA profile with CH_4 emission, which can assist in determining the impact of tanniferous supplement against enteric CH_4 production (212). The medium-chain FA (lauric and myristic acids) have shown a positive correlation with enteric CH_4 production as these FA are synthesized (*de novo* synthesis) in the mammary gland from ruminal acetate and butyrate (213). In this regard, it has been

reported that supplementation of quebracho tannins (30 g/kg DM) could reduce myristic acid content in dairy cows, which reveals the negative effects of tannin on fiber digestibility (214). However, long-chain FA (pentadecanoic and heptadecanoic acid) exhibited a negative correlation with CH_4 emission as their *de novo* synthesis is mediated from ruminal propionate (215). However, further careful investigations are warranted to corroborate this relationship.

Tannins also possess antioxidant properties as they can scavenge free radicals due to hydroxyl groups, degree of polymerization, and redox activities (216–218). Supplementation of tannin has been shown to improve the antioxidant status of cattle and sheep (219–221). Hydrolyzable tannins are considered the most potent antioxidants, which can prevent cellular damage and neutralize free radicals (222), while condensed tannins (catechin) also possess antioxidant activities (223). Pomegranate as hydrolyzable tannin has shown better antioxidant activity as tested on cultured bovine aortic endothelial cells; it has no adverse effects on cell viability and apoptosis. Pomegranate has also exhibited protective effects against membrane lipid peroxidation, owing to its potent ability to reduce the production of intracellular reactive oxygen species (224). However, the optimum level of tannin for antioxidant capacity and its putative mechanism of action in animal tissues require further elucidation. A recent study has revealed that rumen microbial taxa (*Bifidobacterium*, *Lactobacillus*, and *Schwartzia*) exhibited a strong association with host antioxidant capacity and immunomodulatory functions (225).

Prolonged use of the purified form of secondary compounds can lead to antimicrobial resistance; however, tannin supplementation as a crude extract of mixtures (having different molecular sizes) offers a major advantage to control antimicrobial resistance (226). Moreover, studies have shown that CT-rich diets can effectively decrease CH_4 emissions per unit of DMI over a range of dietary CP from 15 to 25%. For example, a decrease up to 25 to 50% was observed in *in vitro* CH_4 production in steers grazing on winter wheat forage (15 to 18% CP) supplemented with quebracho CT extract at 10–20 g/kg DMI (227, 228). This shows the effective inhibitory effects of tannins on ruminal protein degradation and CH_4 emission but requires careful selection of diets and nutrient composition to avoid adverse effects on feed digestibility and efficiency (229). However, further studies are required to fully understand the mechanism of action of tannins regarding modulation of the rumen microbiome, potential inhibitory effects on methanogens and protozoa, and their optimum inclusion levels to elucidate their potential for CH_4 mitigation. Furthermore, focused investigations are required to explore the optimum levels and types of tannins and feeding conditions to reduce GHG emission in commercial ruminant production systems.

Effect of Essential Oils on Rumen Methanogenesis and Fermentation Characteristics

Essential oils are terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoid compounds with characteristic flavors and odors, formed by different plants (herbs and spices). They contain numerous chemical substances, for example, alcohols, hydrocarbons, ketones, aldehydes, ethers, and esters, and mostly EO are lipophilic complexes (230, 231). Various studies have been performed to evaluate the effect of EO on rumen fermentation and feed degradability. Many *in vitro* and *in vivo* trials have proved the favorable effect of EO in reducing CH_4 production and altering microbial populations (**Tables 5**, **6**). The potential effect of EO on rumen fermentation and methanogenesis is mainly mediated by their antimicrobial activities owing to their interaction with cell membranes of microbes (by disrupting membrane stability of lipid bilayer). They are most effective against gram +ve bacteria and possess almost no activity against gram –ve (because of their hydrophilic bilayer) except thymol and carvacrol (232). Garlic oil has shown inhibition of HMG-CoA reductase, leading to membrane instability and, eventually, cell death in methanogenic archaea. Recently, metagenomic analysis of goat rumen revealed that EO cobalt complexes significantly manipulated the structural and functional profile of rumen microbiota. It was revealed that *Bacteroides* sp. and *Succinivibrio* sp. showed a positive correlation with enhanced VFA production in supplemented groups. Moreover, functional prediction pathway analysis exhibited upregulation of lipid and carbohydrate pathways by EO (233).

Some studies have also reported a few unfavorable effects of using EO as feed additives as they depressed synthesis of VFA by reducing feed degradability (234). These harmful effects might be due to their extensive and non-specific antimicrobial properties in the rumen. In a study, no effect on the rumen microbiome has been observed by supplementation of a blend of EO having thymol, guaiacol, eugenol, vanillin, salicylaldehyde, and limonene (235). The desirable effects of EO on the rumen physiology are mainly attributed to their phenolic compounds, which possess the potent ability to affect the activity of both gram-positive and gram-negative bacteria (236). Inhibition of gram-positive bacteria in the rumen can potentially increase the propionate concentration (235). Supplementing a blend of EO (cinnamaldehyde, eugenol, carvacrol, and capsicum oleoresin) firstly increased rumen acetate concentration, which was replaced by propionate concentration afterward. This shift indicated a combined effect of low pH and antimicrobial activity of EO. Furthermore, this blend of EO showed the ability to improve microbial protein synthesis in sheep (237). Higher microbial protein might be attributed to the enhanced post-ruminal protein supply and absorption. Moreover, it might possibly due to the reduction of protozoal counts as ruminal protozoa devour many bacteria and their protein flow toward the small intestine (237). Recently, Garcia et al. (238) revealed that the chemical composition of EO, especially proportion of oxygenated compounds, has a positive interaction with fermentation pattern and indicate promising potential regarding CH_4 mitigation. However, EO have shown inconsistent effects on rumen microbes and feed degradability in different studies, owing to different types of EO used, their chemical composition, and their variable dietary and host responses (**Tables 5**, **6**).

Supplementation of oregano EO at 4 and 7 g/d promoted the population of primary cellulolytic bacteria and ruminal fungi,

TABLE 5 | Effect of various EO and their compounds on rumen microbial population.

Sources	Test system/dose	Diet	Total bacteria	Protozoa	Methanogens	*F.S*	*R.F*	*R.A*	*B.F*	References
Oregano essential oil	*In vitro* (13, 52, 91, and 130 mg/L	F:C (65.5:34.5)	=	NF	NF	↓	=	=	=	(239)
Oregano oil and carvacrol	Cannulated cows (50 mg/kg of DM)	TMR	NF	=	NF	NF	NF	NF	NF	(240)
Oregano essential oil	*In vivo* sheep 4 g/d 7 g/d	F:C (65.5:34.5)	 ↑ =	 ↓ ↓	NF	 ↑ =	 ↑ ↓	 ↑ =	NF	(241)
Essential oil-cobalt	Goat 52 mg/d 91 mg/d	Concentrate	 ↓ ↓	NF	 = =	 ↑ ↓	 ↓ =	 ↓ =	 ↓ =	(233)
Plant-derived EO (carvacrol, eugenol and thymol)	*In vitro* and *vivo* both Control LCP LCP 35 g/d	TMR	NF	= = =	NF	NF	NF	NF	NF	(242)
Mixture of cinnamaldehyde, thymol, and eugenol	Heifer 1 g/kg substrate 2 g/kg substrate	F:C (60:50) 24 h	NF	 = ↑	NF	 = =	 = ↑	 ↑ =	 = ↑	(243)
Thymol:carvacrol	*In vitro* 0:100, 20:80, 40:60, 60:40, 80:20, 100:0	Rumen culture of bovine	 = ↑ ↓ ↑ ↑ ↑	 = ↑ ↓ ↑ ↑ ↑	NF	NF	NF	NF	NF	(244)
Java cardamom	*In vitro* cow 25 mg/l 50 mg/l 75 mg/l 100 mg/l	F:C (60:40)	NF	 = = = =	NF	NF	NF	NF	NF	(245)
Blend of cinnamaldehyde and garlic oil	*In vitro* 0.0043% of DM	F:C (50:50)	NF	=	NF	NF	NF	NF	NF	(246)
Anise EO Anise extract 	*In vitro* rumen buffer 250 μL /30 ml 500 μL /30 ml 750 μL /30 ml 1,000 μL /30 ml 250 μL /30 ml 500 μL /30 ml 750 μL /30 ml 1,000 μL /30 ml	F:C (40:60)	NF	 = = = = = = = =	NF	NF	NF	NF	NF	(247)

F:C., forage to concentrate ratio; TMR, total mixed ration; NF, not found; ↑, increase; ↓, decrease; =, no effect; EO, essential oils; F.S, Fibrobacter succinogenes; R.F, Ruminococcus flavefaciens; R.A, Ruminococcus albus; B.F, Butyrivibrio fibrisolvens; LCP, low CP diet.

respectively, in sheep (241). An *in vitro* study revealed that the inclusion of EO of *S. spicatum* in a high-concentrate diet significantly improved the rumen fermentation characteristics by reducing CH_4 and NH_3-N while promoting propionate concentration (252). Dietary supplementation of EO (coriander, geranyl acetate, and eugenol) reduced CH_4 production up to 6% per cow/day and 20% less CH_4 per kg of milk. It can be speculated that energy saved through this reduced CH_4 production may be diverted toward milk production (253). A blend of EO (carvacrol, caryophyllene, p-cymene, cineole, terpinene, and

TABLE 6 | Effects of various EO and their compounds on methanogenesis, rumen fermentation, and feed degradability.

Sources	Test system/dose	Diet	CH_4	NH_3	tVFA	DMI	Acetate	Butyrate	Isobutyrate	Propionate	Isovalerate	Valerate	Acetate/ Propionate	DMD	References
Oregano essential oil	*In vitro* (13, 52, 91, and 130 mg/L	F:C (65.5:34.5)	↓	↓	↓	NF	↓	↓	↓	↓	↓	↓	↑	↑	(239)
Oregano oil and carvacrol	Cannulated cows (50 mg/kg of DM)	TMR	=	=	=	=	=	=	NF	↑	NF	=	↓	=	(240)
Dried oregano	Dairy cows (18, 36, and 53 g DM/kg of dietary DM in low EO	TMR	=	=	=	=	=	=	NF	=	NF	=	=	=	(248)
Essential oil-cobalt	Goat	Concentrate	NF						NF		NF	NF	NF	NF	(233)
	52 mg/d			↓	↑	=	↑	=		=					
	91 mg/d			↓	=	=	=	=		=					
Lippia turbinate	*In vitro* sheep	F:C (80:20)	↓	=	=	NF	=	=	=	=	=	=	=	↓	(249)
Tagetes minuta	1 ml in fermenter		↓	↓	=		=	=	=	=	=	=	=	↓	
Mix	daily		↓	↓	=		=	=	=	=	=	=	=	↓	
Cashew and	*In vitro* cow	F:C (20:80)	NF						NF		NF	NF			(250)
Castor	1 g/d			=		=	=	=		=			=	=	
	2 g/d			=		=	=	=		=			=	=	
	4 g/d			=		=	=	=		=			=	=	
	8 g/d			=		=	=	=		=			=	=	
Mixture of cinnamaldehyde, thymol, and eugenol	Heifer	F:C (60:50) 24 h	NF						NF		NF	NF			(243)
	1 g/kg substrate			=	=		=	=		=			=	↑	
	2 g/kg substrate			↑	=		=	=		=			=	=	
Thyme	*In vitro* cow 50 μl/l of total culture medium	TMR	NF	↓	NF	NF	NF	NF	NF	NF	NF	NF	NF	↑	(251)
Mint				↓										↑	
Savory				↓										↑	
Lavandula angustifolia	Sheep μl/g DMI	Hig- concentrate diet				NF			NF		NF	NF			(252)
	62.5		↑	↓	↓		=	=		=			=	=	
	125		=	↓	↓		=	↑		↓			↑	=	
	250		↓	↓	↓		↑	=		↓			↑	↓	
	500		↓	↓	↓		=	↓		↓			↑	↓	
Santalum spicatum	62.5		↓	↓	=		=	=		↑			↓	=	
	125		↓	↓	=		=	=		↑			↓	=	
	250		↓	↓	=		=	=		↑			↓	=	
	500		↓	↓	=		=	=		↑			↓	↓	

(Continued)

TABLE 6 | Continued

Sources	Test system/dose	Diet	CH_4	NH_3	tVFA	DMI	Acetate	Butyrate	Isobutyrate	Propionate	Isovalerate	Valerate	Acetate/ Propionate	DMD	References
Thymol:carvacrol ratio	*In vitro* 0:100	Rumen culture of bovine	=	=	NF	NF	NF	NF	NF	NF	NF	NF	NF	=	(244)
	20:80,		=	↑										=	
	40:60,		=	↓										=	
	60:40,		=	↑										=	
	80:20,		=	↑										=	
	100:0		=	↑										=	
EO	Dairy cow 1 g/d	TMR	↓	NF	NF	↑	NF	NF	NF	NF	NF	NF	NF	NF	(253)
Blend of EO (cresols, thymol, limonene, vanillin, guaiacol, eugenol, and salicylate)	*In vitro* cow 20 ml/l	F:C (60:40)	=	=	=	NF	=	=	=	=	=	=	=	=	(254)
	100 ml/l		=	=	=		=	=	=	=	=	=	=	↓	
	200 ml/l		=	=	=		=	=	=	=	=	=	=	=	
	600 ml/l		↓	=	↓		=	↑	↓	↓	=	↓	=	↓	
	1,000 ml/l		↓	↓	↓		↓	↑	↓	↓	↓	↓	↑	↓	
Lemon grass EO	Lamb, 1 ml/kg of DM	F:C (15:85)	NF	NF	=	=	=	↓	↓	=	↓	↑	NF	=	(255)
Java cardamom	*In vitro* cow 25 mg/l	F:C (60:40)	=	=	=	NF	=	=	NF	=	NF	NF	=	↓	(245)
	50 mg/l		=	=	=		=	=		=			=	=	
	75 mg/l		=	↓	=		=	=		=			=	=	
	100 mg/l		=	=	=		=	=		=			=	=	
Plant-derived EO (carvacrol, eugenol and thymol)	*In vitro* and in vivo both	TMR	NF	=	=	↑	=	=	=	=	=	=	=	=	(242)
	Control			=	=	↓	=	=	=	=	=	↓	=	=	
	LCP			=	=	↓	=	=	=	=	=	=	=	=	
	LCP 35 g/d														
Blend of cinnamaldehyde and garlic oil	Sheep 0.0043% of DM	F:C (50:50)	=	=	=	=	=	=	=	=	↑	=	=	=	(246)
Microencapsulated blend of EO	Sheep	TMR													(237)
	0.02%		↓	=	↑	=	=	↑	=	=	=	=	=	=	
	0.04%		↓	=	↑	=	=	↑	=	↑	=	=	↓	=	
Citrus essential oils	*In vitro* 0.8 mL/L rumen volume	TMR (3 weeks)	=	↓	↓	=	↓	=	NF	=	NF	NF	=	=	(256)

F:C, forage to concentrate ratio; TMR, total mixed ration; tVFA, total volatile fatty acid; DMD, dry matter degradability; DMI, dry matter intake; NF, not found; ↑, increase; ↓, decrease; =, no effect; EO, essential oils; LCP, low CP diet.

thymol) altered the rumen functions by selectively promoting the growth of rumen bacteria (by decreasing *Firmicutes* while increasing *Bacteroidetes*) in calves (257). Essential oils of *Lippia turbinata* and *Tagetes minuta* have shown a 10-fold decrease of *in vitro* CH_4 production coupled with modification of N metabolism in the rumen (249). Recently, a meta-analysis showed that dietary supplementation of a blend of EO (coriander, eugenol, and geraniol) increased the milk yield (3.6%), milk fat and protein (4.1%), and feed efficiency (4.4%), while decreasing DM intake (12.9%) and CH_4 production (8.8%) during long term trial in dairy cattle (258).

Essential oil–cobalt complexes have shown positive effects in ruminants by enhancing productive performance while decreasing NH_3 emissions (233). Likewise, synergetic effects of EO (thyme, mint, and savory) in a high-concentrate diet have been observed regarding desirable shifts in microbial fermentation and higher microbial protein yield in dairy cows (251). Increased feed efficiency and calcium homeostasis have been observed with supplementation of a plant bioactive EO blend (>80% menthol, eugenol, and anethol). Increased uptake of calcium and ammonium was also observed as a result of specific cation-transporting proteins expressed by the rumen. However, further investigations are needed to evaluate the fate of the absorbed nutrients, especially calcium and N (259). Recently, Zhou et al. (239) suggested that oregano EO (52 mg/L) in mature ruminants can modify ruminal fermentation and mitigate *in vitro* CH_4 production through mediating ruminal bacteria (*Prevotella* and *Dialister*). Some studies involving supplementation of EO in ruminants have shown contrary findings as the feeding of oregano EO did not reduce CH_4 yield together with no effect on animal performance and rumen fermentation (240, 248). These divergent findings may be partially explained by variable experimental conditions of studies including the type of diets, plant species, dose and type of EO, pH of rumen fluid, and host animal (260, 261).

Studies have suggested the use of a combination or blend of different EO as a better strategy to modulate rumen microbiome to manipulate rumen fermentation than using individual EO. This is mainly because each EO possesses a complex mixture of phytochemicals and their synergistic effects can lead to the synthesis of new compounds with a quite different bioactivity that could not be harvested with individual compounds (29, 262). Additionally, using a combination of phytochemicals is also advantageous for the host regarding the provision of various phytonutrients from different plant combinations. Moreover, the benefits of such a combination are its ultimate utility for using on large scale in the animal industry as a commercial feed additive to have an overall impact on the improvement of global animal production while mitigating GHG emissions.

FUTURE IMPLICATIONS

Rumen microbiome plays a critical functional role in N_2 utilization, rumen feed fermentation, and CH_4 production, ultimately influencing the production, health, and welfare in ruminants. Rumen microbes are highly active and can adapt to an extensive range of dietary fluctuations or host physiological conditions. Extensive literature supports the supplementation of phytogenic feed additives like saponin, tannins, and EO for the manipulation of rumen microbiome to modulate ruminal fermentation to increase VFA and decrease NH_3 and CH_4 production. Decreasing methanogenesis using dietary interventions at the expense of decreased VFA production is nutritionally adverse and unadvisable. Inhibition of enteric CH_4 emission in ruminants is possible through the use of plant bioactive compounds; however, studies on the long-term effects of these compounds to reduce methanogenesis are essentially required. Studies summarized above clearly demonstrate that although phytochemicals possess a potent ability to modulate rumen microbiome and reduce methanogenesis *in vitro*, the observed *in vivo* effects varied greatly. Many factors, including variations of the chemical compositions of the compounds due to the differences in plant origin, growing conditions, and processing methods as well as different application methods, feeding conditions, and progressive adaptation of microbes for specific phytochemicals, contribute to this vast variability. Because of the complexity of these issues, it is difficult to conduct systematic and comprehensive evaluations of the efficacy and safety of these compounds for commercial applications in the animal industry. Therefore, controlling this variability is key to developing phytogenic substances as natural feed additives. This ideally should include all procedures from production, extraction, processing, and application. Optimization of different conditions during these steps can definitely help to address problems like inconsistency and transient and adverse effects of phytogenic feed additives in ruminants. Recent developments in molecular docking analysis and three-dimensional structure databases of phytochemicals have opened a new horizon for the discovery of putative functions (particularly antimicrobial and antimethanogenic) of different compounds by evaluating the structure affinity relationships with different microbes and their substrates. It will be of interest to identify potent phytochemicals based on their structural homology and binding affinity with functional proteins of rumen microbes particularly methanogenic archaea through molecular docking analysis first and then testing their biological activity *in vitro* and *in vivo*. This approach will not only help to find out new phytochemicals with potent activities but also help to understand their mechanism of action and exploit their synergistic effects and interactions with other compounds. Moreover, there is a dire need to exploit advances in molecular chemistry like encapsulation techniques to avoid ruminal degradation of phytochemicals and use their nanostructures to enhance their bioactivity and bioavailability, which seems to be an exciting area to explore their promising effects on the rumen microbiome.

It has been proposed that higher molecular weight compounds (such as polyphenols) were not dissolved well in water (263). Nanoparticles are formulated with hydrophobic groups inside and polar groups on the surface of particles and have shown to significantly enhance the solubility and bioavailability of less-water-soluble phenolic phytochemicals (264). Moreover, nanoparticles of plant extracts and EO have shown higher antioxidant and antimicrobial activities as compared with their crude extracts or EO (265). The higher antimicrobial activity of the nanoparticles or nanoemulsion of phytochemicals is related

to the size of the nanoparticles or nanoemulsion droplets, which is in the subcellular size range. This allows the penetration of the nanoparticles or nanoemulsion droplets to the microbial cells leading to enhanced activity (265, 266). It is anticipated that the use of nanoparticles and nanoemulsions can potentially enhance the modulatory effects of phytochemicals on the rumen microbiome, subsequently leading to better health and performance of ruminants.

AUTHOR CONTRIBUTIONS

FH and CY: conceptualization. MAA, SS, and HME: data curation. CY: funding acquisition, project administration, supervision, and validation. FH, MSK, and MSR: investigation. MSR and CY: resources. FH and MSR: software. MAA, MSR, MSK, and FH: writing—original draft and formal analysis. MAA, FH, HME, and CY: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Radix Puerarin Extract (*Puerarin*) Could Improve Meat Quality of Heat-Stressed Beef Cattle Through Changing Muscle Antioxidant Ability and Fiber Characteristics

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The experiment was conducted to investigate the effects of dietary supplementation with *Puerarin* on meat quality, muscle antioxidant ability, and muscle fiber characteristics of beef cattle under a hot environment in summer. Thirty-two 15 ± 1.5-month-old Jinjiang bulls (291.65 ± 8.84 kg) were randomly divided into four groups with dietary *Puerarin* at 0 (control), 200 (Pue200), 400 (Pue400), and 800 (Pue800) mg/kg in the feed concentrate ($n = 8$). The feeding trial lasted for 60 days after a 10-day adaptation period (July 1–September 8); the average values of temperature, relative humidity and temperature, and humidity index were 30.68°C, 68.05%, and 81.81, respectively. The growth performance on day 40 of the experiment period was calculated. After 60 days' experimental period, four Jinjiang cattle per treatment from the control group, Pue400 group, and Pue800 group were slaughtered. Compared with the control group, the Pue400 and Pue800 groups improved the growth performance of beef cattle; the Pue800 group elevated the activities of superoxide dismutase, total antioxidant capacity, and glutathione peroxidase in the *M. longissimus thoracis* (LT) muscle. In the control group, the cell membrane was incomplete, and most of the mitochondria were elongated and in a fission state, while in the Pue400 and Pue800 groups, the cell membrane was clear and complete, and the mitochondria presented with round and oval shapes. Compared with the control group, the Pue400 and Pue800 groups reduced the shear force of the LT muscle, and the Pue400 group decreased the muscle fiber diameter and the myosin heavy-chain (MyHC)-IIb gene expression. Furthermore, the Pue400 and Pue800 groups decreased the ratio of AMP/ATP, the Pue800 group reduced the AMP-activated protein kinase α2 mRNA expression, and the Pue400 group improved the nuclear respiratory factor 1 mRNA expression. These results indicated that dietary supplementation with *Puerarin* might be beneficial to the meat quality of heat-stressed beef cattle by improving muscle antioxidant ability and reducing the MyHC-IIb muscle fiber composition. Based on the results of this study, we recommended 400 mg/kg *Puerarin* in the feed concentrate of beef cattle (~300 kg) for mitigation of heat stress.

Keywords: *Puerarin*, beef cattle, heat stress, meat quality, muscle fiber type

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INTRODUCTION

Heat stress caused by high temperature is one of the most critical environmental stressors challenging cattle production (1), particularly during the hot-humid season in subtropical regions such as South China. The oxidative stress induced by heat stress can impair cell membrane and mitochondrial integrity through lipid peroxidation, which adversely affects the muscle protein functionality and the sensory, nutritional, and shelf-life quality of animal products (2, 3). Moreover, muscle fiber is an important part of the muscle; the quantity and type of muscle fiber are the keys to determine meat quality. Generally, muscle fiber is classified into four categories: myosin heavy-chain (MyHC)-I (slow-oxidative), MyHC-IIa (fast-oxidative), MyHC-IIx (intermediary to MyHC-IIa and IIb), and MyHC-IIb (fast-glycolytic) (4). They can influence meat quality due to their different activities of glycolytic enzymes and contents of glycogen and lipid (5). Notably, previous studies showed that acute or chronic heat stress resulted in decreased slow muscle fiber composition and ultimately led to poor meat quality (6, 7). Therefore, heat stress mitigation is a significant demand for beef cattle welfare and an important guarantee for high-quality beef production.

Unraveling the heat stress-responsive mechanisms in beef cattle is of great importance to the development of active agents against heat stress. AMP-activated protein kinase (AMPK) is a serine/threonine kinase that plays a crucial role in regulating cellular and whole-body energy homeostasis under physiological stress conditions (8). When the body suffers from heat stress, ATP consumption increases, subsequently resulting in elevation of AMP/ATP ratio and further activating AMPK *via* phosphorylation of αThr^{172} (9, 10). AMPK can also be activated by cellular reactive oxygen species (ROS) and intracellular calcium (11). Once activated, AMPK phosphorylates a range of substrates to preserve energy expenditure by switching from anabolic to catabolic pathway (8). Among all the AMPK-mediated regulation pathways of energy homeostasis maintenance in response to stress, what meat scientists valued most is the regulation of skeletal muscle energy metabolism (3). Moreover, the activation of AMPK has been proven to cause muscle to assume a faster-contracting and more glycolytic property (12). Therefore, regulating the activity of AMPK may effectively relieve the negative impact of heat stress on beef quality.

Puerarin is a natural active isoflavone extracted from the traditional Chinese medicine (TCM) *Radix Puerarin* and has a wide range of functions, including anti-oxidative, anti-inflammation, and anti-apoptosis (13, 14). It has been reported that *Puerarin* could attenuate heat stress by suppressing ROS production and upregulating heat shock protein-72 expression in bovine Sertoli cells (15). Besides, *Puerarin* could alleviate autophagy by inhibiting the AMPK–mammalian target of rapamycin (mTOR)–Unc 51 like autophagy activating kinase 1 (ULK1) signaling pathway (16). A previous study showed that *Puerarin* was used as a superior animal feed additive, natural health food, and effective medicine in Asian countries (17). However, little attention has been paid to *Puerarin*'s improvement effects on the meat quality of heat-stressed beef cattle, and whether this improvement is associated with the changes in muscle antioxidant ability and muscle fiber characteristics is still unclear. Therefore, this experiment was intended to study the effects of *Puerarin* on the meat quality, muscle antioxidant ability, muscle fiber characteristics, and AMPK pathway of beef cattle under high temperature in summer.

MATERIALS AND METHODS

This experiment was approved by the Committee for the Care and Use of Experimental Animals at Jiangxi Agricultural University (JXAULL-20190015).

Preparation of *Puerarin*

Puerarin preparation (purity N 98.1%) was provided by the Department of Pharmaceutical Chemistry, Guangxi Medical University (Nanning, China). *Puerarin* is a white powder extracted from *R. Puerarin*. In these experimental diets, *Puerarin* was first mixed with the premix in a certain proportion and then formulated into a concentrate.

Animal Treatments and Experimental Diets

Thirty-two 15 ± 1.5-month-old healthy and uncastrated Jinjiang bulls (291.65 ± 8.84 kg) were randomly divided into four groups. Jinjiang cattle are a breed of Chinese indigenous beef cattle that are bred in the northwest of the Jiangxi Province. Dietary treatments were basal diet without any additive (control), basal diet + 200 mg/kg *Puerarin* in the feed concentrate (Pue200), basal diet + 400 mg/kg *Puerarin* in the feed concentrate (Pue400), and basal diet + 800 mg/kg *Puerarin* in the feed concentrate (Pue800). Each treatment consisted of eight replicates (pen) with one beef cattle per pen. Animals were housed indoors with solid concrete floor pens (1.25 m × 2 m). The feeding trial lasted for 60 days after a 10-day adaptation period (July 1–September 8; the average values of temperature, relative humidity and temperature, and humidity index were 30.68°C, 68.05%, and 81.81, respectively). According to the Chinese Feeding Standard for beef cattle (NY/T 815-2004), the basal diet was designed and met the nutrient requirements of 0.5 kg daily gain of 300 kg male cattle. The ingredient composition and nutrient levels of the basal diet were shown in **Table 1**. In this study, the level of *Puerarin* supplemented in the basal diet was referred to the study of Zhao et al. (18), who reported that the crossbred steers (Chinese Yellow × Augus, 565.2 ± 31.2 kg) fed a 90% concentrate diet supplemented with daidzein (500 mg/kg concentrate). *Puerarin* is a structural analog of daidzein. Based on these data, it can be calculated that the equal amount of *Puerarin* is 908 mg/kg basal diet in 565 kg of beef cattle. Therefore, we hypothesized that 291 kg of Jinjiang cattle could be fed 468 mg/kg *Puerarin* in basal diet. No antibiotic was included in the diets. The diet was provided twice daily (06:00, 15:30). All animals were offered feed and water *ad libitum*.

Growth Performance

Due to this study being conducted to investigate the effects of heat stress on meat quality of beef cattle, we recorded final body weight

TABLE 1 | Composition and nutrient levels of the basal diet (air-dry basis, %).

Ingredients	Content	Nutrient levels	Content
Rice straw	20	DM	89.42
Brewer's grains	20	CP	11.19
Wheat	56.5	Ash	7.80
NaCl	0.5	NDF	30.08
$NaHCO_3$	1.0	ADF	15.18
Premix[a]	2.0	NE_{mf}/(MJ/kg)[b]	5.45
Amount	100.0	P	0.67
		Ca	1.11

[a]*The premix provided per kilogram of diet: 3,200 mg of iron as iron sulfate, 1,500 mg of manganese as manganous oxide, 2,000 mg of zinc as zinc oxide, 650 mg of copper as copper sulfate, 35 mg of iodate as calcium iodate, 10 mg of selenium as sodium selenite, 10 mg of cobalt as cobalt chloride, 130 g of calcium as calcium carbonate, 30 g of phosphorus as calcium hydrogen phosphate, 45 mg retinyl acetate, 40 μg cholecalciferol, and 3.0 mg DL-α-tocopheryl acetate.*

[b]NE_{mf} *were calculated values, while others were measured values. DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NEmf, combined net energy; P, phosphorus; Ca, calcium.*

on day 40 of the experiment period to avoid the pre-slaughter stress induced by weighing. Therefore, the initial body weight (on the first day of the experiment period), final body weight (on day 40 of the experiment period), and dry matter intake of beef cattle in each replicate were recorded to calculate average daily gain (ADG), average dry matter intake (ADMI), and the ratio of feed to gain (F/G). ADG = (final body weights per replicate – initial body weights per replicate)/40 days, ADMI = dry matter intake per replicate/40 days, F/G = ADMI/ADG.

Sample Collection

At the end of the experiment, based on the earlier period data (day 1–40 of the experiment period) of growth performance, we found that the Pue200 group reduced the ADG and ADMI of beef cattle numerically compared with the control group, while the Pue400 and Pue800 groups significantly improved the ADG and ADMI of beef cattle. Therefore, four beef cattle per treatment with one cattle per pen with medium body weight were selected from the control, Pue400, and Pue800 groups for further analyses. A total of 12 beef cattle were gently led to the slaughterhouse on foot to avoid stress (the distance between feedlot and slaughterhouse was 0.7 km). At the day of slaughter, the average temperature and humidity index (THI) was 78.3.

All animals were sacrificed *via* electrical stunning, followed by jugular vein exsanguination. Immediately, the *Musculus longissimus thoracis* (LT) muscle sampled at the last rib of the left carcass was placed in liquid nitrogen to analyze the antioxidant index, adenosine phosphates, and the expression of genes mRNA. Then, the LT muscle samples were cut into shaped strips (1 cm × 1 cm × 1 cm), parallel to the muscle fiber direction, and placed in a 4% formaldehyde solution for the analysis of muscle fiber morphology. The other LT muscle samples were cut into shaped strips (1 mm × 1 mm × 1 mm), parallel to the muscle fiber direction, placed in a pre-cooled 2.5% glutaraldehyde for 2 h, and stored in the refrigerator at 4°C until for the analysis of skeletal muscle ultrastructure. At 30-min postmortem, the LT muscles from the last rib of the left carcass were removed, trimmed of subcutaneous fat and connective tissue, and vacuum-packed at 4°C for the measurement of meat quality.

Antioxidant Index Analyses

For biochemical assays, 0.5 g frozen LT muscle samples were ice bath homogenized in 4.5 ml ice-cold physiological saline for 1 min and then centrifuged (2,700 g, 4°C, 10 min). The supernatants were then subjected to the measurements of the content of malondialdehyde (MDA), the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), the total antioxidant capacity (T-AOC). The concentration of total protein in the LT muscle was determined by TP kit (Nanjing Jiancheng Bioengineering Institute, Nangjing, China) according to the instructions of the manufacturer. All of those antioxidant indices in the LT muscle were determined by commercial MDA, SOD, GSH-PX, and T-AOC kits (Nanjing Jiancheng Bioengineering Institute) and were normalized by total protein concentration in the LT muscle, respectively, according to the instructions of the manufacturer. The ROS content was determined by ROS kit (Nanjing Jiancheng Bioengineering Institute, Nangjing, China) according to the instructions of the manufacturer. The 2′,7′-dichlorofluorescin diacetate (DCFH-DA) is the most commonly used sensitive ROS probe.

Meat Quality

Muscle pH_{45min} and pH_{24h} were measured by using a pH electrode (HI99163N, Hanna, Padova, Italy). Each chop was measured three times at different areas, and the average value was obtained.

Meat color was measured using a spectrocolorimeter (WSC-S, Shanghai, China) at 24 h postmortem. Mean CIE L* (lightness), a* (redness), and b* (yellowness) values were collected from three different locations of the chops using freshly cut surface after being exposed to air for 20 min.

For the determination of cooking loss, at 48 h postmortem, samples of LT muscle (6 cm × 6 cm × 4 cm) were vacuum-packed in individual polyethylene vacuum bags and cooked in a water bath at 80°C to reach an internal temperature of 70°C. Then, the samples within their packaging bags were cooled in running water to room temperature, wiped with absorbent paper to remove residual moisture, and reweighed to calculate cooking loss. Then, the cooked samples were cut into shaped strips (1 cm × 1 cm × 3 cm), parallel to the muscle fiber direction. Warner–Bratzler shear force was measured using a C-LM3B shear apparatus (Northeast Agricultural University, Harbin, China) with a load cell of 15 kg and a crosshead speed of 200 mm/min. Six replicates of each sample were measured.

Muscle Fiber Morphology

The LT muscle samples in 4% formaldehyde solution were taken out and then dehydrated, paraffin-embedded, sliced, hematoxylin–eosin-stained, and sealed. The sections were taken photographs with a microscope (Motic BA210; Motic Medical Diagnostic Systems, Co., Ltd., Xiamen, China) at a magnification of 400×. Motic Images Advanced 3.2 software was used to analyze pictures, including muscle fiber diameter and density. Five views were captured in each section.

Transmission Electron Microscopy

The LT muscle samples in 2.5% glutaraldehyde were taken out and then washed with 0.1 mol/L phosphate buffer followed by postfixation of 1% osmium tetraoxide. Samples were then washed with 0.1 mol/L phosphate buffer again, dehydrated by gradient of increasing concentrations of alcohol, embedded with epon resin, and sliced. Transmission electron microscopy (TEM) images were obtained on a Hitachi H-7100 transmission electron microscope.

Adenosine Phosphate Analyses

The levels of adenosine phosphates (ATP, ADP, and AMP) in the LT muscle were determined by high-performance liquid chromatography (HPLC) according to the method of Li et al. (19). Briefly, 0.5 g of frozen muscle sample was homogenized in 2.5 ml of 7% ice-cold perchloric acid at 13,500 rpm for 30 s in an ice bath and then centrifuged (15,000 × g, 4°C, 10 min). The supernatant (850 μl) was then neutralized with 0.85 M KOH (850 μl) and centrifuged again (15,000 × g, 4°C, 10 min) to remove $KClO_4$. The neutralized supernatant was filtered through a 0.45-μm filter before injection into a Waters-2695 Alliance HPLC system (Waters, Milford, MA, USA). The column was a Kromasil 5 μm C_{18}, 250 × 4.6 mm (Feinano, Tianjin, China). The chromatographic conditions were as follows: mobile phase A, HPLC grade methanol; mobile phase B, phosphate buffer (2.5 mM tetra-butylammonium hydrogen sulfate, 0.04 M potassium dihydrogen orthophosphate, 0.06 M dipotassium hydrogen orthophosphate, pH 7.0), filtered through a 0.45-μm membrane; mobile A/mobile B, 13.5%/86.5%. The column temperature was set at 30°C, the injection volume was 10 μl, and UV detection was at 254 nm. The characteristic running time was 15 min, the flow rate was maintained at 1.0 ml/min, and sample measurement was set to auto-sequence injection. Peaks were identified and quantified using standard curves.

Total RNA Isolation and mRNA Expression Analyses

Total RNA was isolated from the LT sample using the phenol and guanidine isothiocyanate-based TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The purity and quantity of total RNA were measured by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at 260 and 280 nm. Total RNA was treated with DNase I (Takara Biotechnology Co. Ltd., Dalian, China) to remove DNA and transcribed to cDNA using a PrimeScript RT Master Mix kit (Takara Biotechnology Co. Ltd., China) following the manufacturer's instructions. The mRNA expressions were determined and calculated according to the method of Li et al. (20). Briefly, real-time PCR was carried out in optical 96-well plates on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex Taq Kit (Takara Biotechnology Co., Ltd., China). Primers used for real-time PCR are presented in **Table 2** and were synthesized by Invitrogen. The amplification was performed in a total volume of 20 μl, containing 10 μl of SYBR Premix Ex Taq, 0.4 μl of each primer (10 μM), 6.8 μl of sterilized double-distilled water, and 2 μl of cDNA. The program was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 58°C for 31 s, and 70°C for 30 s, and collected the fluorescence signal at 58°C. The amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes was used for each sample to normalize the expression of the selected genes. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

TABLE 2 | The primer sequences of genes.

Gene	Primer sequence (5′-3′)	Number	Product size (bp)
LKB1	F: CACCGAGGTCATCTACCAGC	XM_024995125.1	114
	R: GAGTCCAGCACCTCCTTCAC		
AMPKα2	F: TGAGAAGCAGAAGCACGACG	NM_001205605.1	113
	R: GGCCTGTCAATTGATGCTCT		
PGC-1α	F: TGCAGTACACATCAGCCTCA	NM_177945.3	95
	R: TGCCAGGAGTTTGGTTGTGAT		
Nrf1	F: AAACTGGGCCACGTTACAGG	NM_001098002.2	175
	R: TTTTATTGCCCACCCCTGCC		
MyHC-I	F: CAAGGAGCTTCAGGCACGTA	NM_174727.1	179
	R: CGCGCTTCTTGTTCATCTCG		
MyHC-IIa	F: AGACTCTCAAGAGGGACGCT	XM_010816053.3	77
	R: CCTGGAAGTGAGACGGTTCC		
MyHC-IIx	F: CTGAGGAACGGGCTGACATT	NM_174117.1	164
	R: AGTACAAAACAGAGTGACAAAGATT		
MyHC-IIb	F: GTCGGGCTGTACCAGAAGTC	XM_002695806.5	74
	R: CCCTCTTCAGCACTTGGACC		
GAPDH	F: GAAGGTCGGAGTGAACGGAT	NM_001034034.2	180
	R: TTCTCTGCCTTGACTGTGCC		

LKB1, receptor serine/threonine kinase 1; AMPKα2, AMP-activated protein kinase α2; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; Nrf1, nuclear respiratory factor 1; MyHC, myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analyses

All data analyses were statistically analyzed by one-way ANOVA with SPSS statistical software (Ver. 20 for windows, SPSS). All data were normally distributed *via* the Shapiro–Wilk test. Tukey multiple range test was used to compare differences among the treatment groups. The level of statistical significance was present at $P < 0.05$. Values were expressed as mean ± SE.

RESULTS

Growth Performance

As shown in **Table 3**, compared with the control and Pue200 group, dietary supplementation with 400 and 800 mg/kg *Puerarin* improved the ADG of beef cattle ($P < 0.05$). The ADMI of beef cattle in the Pue800 group was higher than that in the control group ($P < 0.05$). However, there was no difference in the initial and final body weights and F/G of cattle among all treatments.

Antioxidant Index

As presented in **Table 4**, the Pue800 group tended to reduce the level of ROS compared with the control group ($P = 0.051$). The SOD activity in the Pue400 and Pue800 groups was higher than

TABLE 3 | Effects of *Puerarin* on growth performance of beef cattle under hot environment.

Item	Groups				*P*-value
	Control	Pue200	Pue400	Pue800	
Initial body weight (kg)	298.86 ± 14.34	294.50 ± 21.46	280.40 ± 25.36	290.86 ± 16.54	0.915
Final body weight (kg)	318.43 ± 14.32	310.80 ± 16.45	308.60 ± 23.16	318.29 ± 7.90	0.965
ADG (kg/day)	0.49 ± 0.02^{b}	0.41 ± 0.03^{b}	0.71 ± 0.07^{a}	0.69 ± 0.07^{a}	0.002
ADMI (kg/day)	4.53 ± 0.25^{bc}	4.02 ± 0.24^{c}	5.41 ± 0.39^{ab}	5.70 ± 0.30^{a}	0.003
F/G (kg/kg)	9.34 ± 0.63	10.05 ± 0.68	7.85 ± 0.58	8.59 ± 0.51	0.125

^{a,b}Means within a row with no common superscript differ significantly ($P < 0.05$).
All traits in this table were analyzed with cattle as the experimental unit ($n = 8$).
ADG, average daily gain; ADMI, average dry matter intake; F/G, feed-to-gain ratio.

TABLE 4 | Effects of *Puerarin* on antioxidant index in *M. longissimus thoracis* of beef cattle under hot environment.

Item	Groups			*P*-value
	Control	Pue400	Pue800	
ROS (fluorescence value/mg prot)	52.72 ± 1.51	51.60 ± 3.17	43.07 ± 2.72	0.051
SOD (U/mg prot)	6.88 ± 0.31^{b}	8.36 ± 0.27^{a}	8.34 ± 0.40^{a}	0.017
T-AOC (U/mg prot)	0.87 ± 0.04^{b}	0.99 ± 0.08^{ab}	1.13 ± 0.06^{a}	0.042
GSH-PX (U/mg prot)	55.98 ± 1.69^{b}	56.65 ± 2.55^{b}	66.23 ± 2.42^{a}	0.018
MDA (nmol/mg prot)	0.48 ± 0.02	0.42 ± 0.02	0.43 ± 0.03	0.176

^{a,b}Means within a row with no common superscript differ significantly ($P < 0.05$).
All traits in this table were analyzed with cattle as the experimental unit ($n = 4$).
ROS, reactive oxygen species; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; GSH-PX, glutathione peroxidase; MDA, malondialdehyde.

TABLE 5 | Effects of *Puerarin* on the meat quality in *M. longissimus thoracis* of beef cattle under hot environment.

Item	Groups			*P*-value
	Control	Pue400	Pue800	
pH_{45min}	6.59 ± 0.07	6.60 ± 0.21	6.61 ± 0.10	0.991
pH_{24h}	5.42 ± 0.05	5.68 ± 0.01	5.55 ± 0.15	0.269
Lightness, L*	0.46 ± 0.04	0.51 ± 0.18	0.43 ± 0.03	0.606
Redness, a*	3.07 ± 0.45	2.91 ± 0.38	3.09 ± 0.38	0.793
Yellowness, b*	1.07 ± 0.02	1.19 ± 0.18	0.83 ± 0.09	0.745
Cooking loss (%)	21.78 ± 4.79	18.14 ± 5.09	17.07 ± 3.21	0.335
Shear force (N)	80.97 ± 0.77^{b}	58.83 ± 5.84^{a}	75.28 ± 1.48^{a}	0.014

^{a,b}Means within a row with no common superscript differ significantly ($P < 0.05$).
All traits in this table were analyzed with cattle as the experimental unit ($n = 4$).

that in the control group ($P < 0.05$). In comparison to the control group, the Pue800 group increased the activities of T-AOC and GSH-PX in the LT muscle ($P < 0.05$). However, an insignificant effect was presented on the MDA content.

Transmission Electron Microscopy

As shown in **Figure 1**, in the control group, the skeletal muscle cell boundaries were not clearly visible and had an incomplete membrane. Besides, the mitochondrial structure was deformed, most of the mitochondria were elongated and in a fission state. In the Pue400 and Pue800 groups, the cell membrane was clear and complete, and the mitochondrial structure was standard, which presented with round and oval shapes. The mitochondrial cristae in the Pue400 group were normal with clear structures, whereas this was not the case in the Pue800 group.

Meat Quality

As shown in **Table 5**, diet supplemented with 400 and 800 mg/kg *Puerarin* reduced the shear force of LT muscle compared with the control group ($P < 0.05$), while there was no significant difference in pH_{45min} value, pH_{24h} value, lightness (L*) values, redness (a*) values, yellowness (b*) values, and cooking loss of the LT muscle among groups.

Muscle Fiber Morphology

As shown in **Figure 2**, diet supplemented with 400 mg/kg *Puerarin* reduced the diameter (**Figure 2B**) of the LT muscle compared with the control group ($P < 0.05$). However, there was no difference in the muscle fiber density (**Figure 2C**) among groups.

Muscle Fiber Type

As shown in **Figure 3**, diet supplemented with 400 mg/kg *Puerarin* reduced the MyHC-IIb gene expression compared with the control group ($P < 0.05$). No difference was noticed about the mRNA expression of MyHC-I, MyHC-IIa, and MyHC-IIx among treatments.

Adenosine Nucleotides

As presented in **Table 6**, diet supplemented with 400 mg/kg *Puerarin* improved the content of ATP compared with the control group ($P < 0.05$), and the ratio of AMP/ATP in the Pue400 and Pue800 groups were lower than those in the control group ($P < 0.05$). However, no difference was noticed about the contents of ADP and AMP among groups.

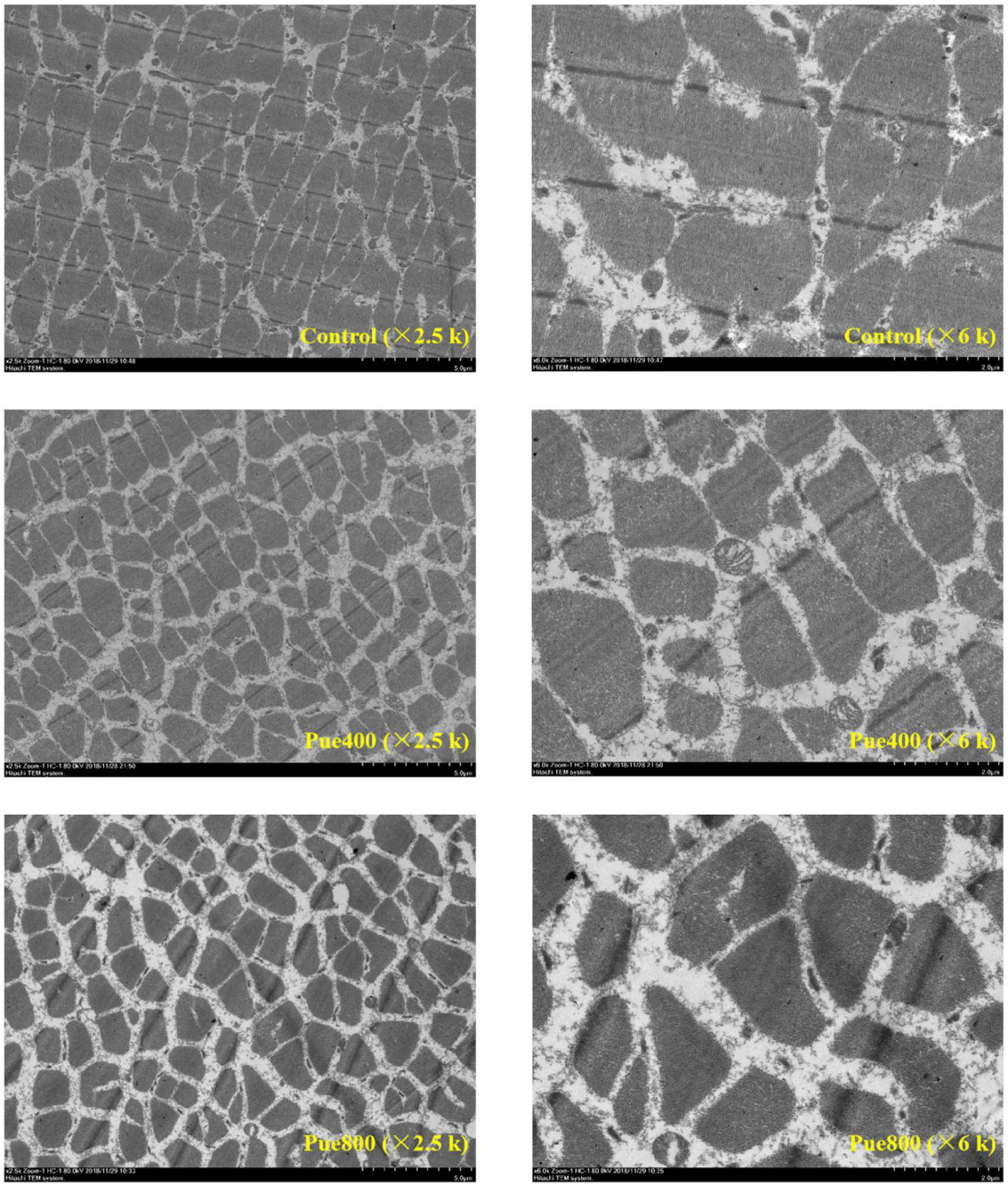

FIGURE 1 | Effects of *Puerarin* on the skeletal muscle ultrastructure in *M. longissimus thoracis* of beef cattle under hot environment.

AMPK Signal Pathway-Related Gene Expressions

As shown in **Figure 4**, compared with the control group, the Pue800 group reduced the mRNA expression of AMPKα2 ($P < 0.05$). Moreover, the Pue400 group improved the mRNA expression of nuclear respiratory factor 1 (Nrf1) ($P < 0.05$). However, an insignificant effect was presented on the receptor serine/threonine kinase 1 [liver kinase B1 (LKB1)] and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) gene expression.

DISCUSSION

The results of our previous study showed that the average daily THI values during the experimental period were higher than 79 for 54 out of 60 days (unpublished data), which indicated

that the experimental beef cattle were in a state of heat stress according to the report of Livestock Conservation, Inc. (LCI) (21). It is widely known that heat stress leads to the decline of production performance, which has been suggested to be partly caused by the reduction of dry matter intake (22). In this study, diet supplemented with *Puerarin* improved the ADG of beef cattle, which might be partly attributed to the increased ADMI induced by *Puerarin* supplementation.

Numerous studies show that farm animals exposed to a hot environment are also related to an occurrence of oxidative stress and further result in a disorder of free radical metabolism and excessive production of ROS (2, 23). Subsequently, the high level of muscle ROS may increase the risk of oxidative reactions during the processes involved in the conversion of muscle into meat. One previous study found that pretreatment with *Puerarin* protected bovine Sertoli cells (bSCs) from heat stress by suppressing ROS production and changing the SOD, catalase (CAT), and GSH-PX activities and MDA content (15). Another study reported that *Puerarin* supplementation reduced the level of ROS and increased the activities of SOD and CAT in the kidney of diabetic mice (24). Similarly, in this study, dietary supplementation with *Puerarin* markedly increased the activities of SOD, GSH-PX, and T-AOC and reduced the content of MDA in the LT muscle of heat-stressed beef cattle. There was little research conducted on the effect of *Puerarin* on the antioxidant capacity of beef cattle. For ruminant, the isoflavones are readily hydrolyzed by rumen microbes, and the major metabolic transformation of isoflavones is performed in the

TABLE 6 | Effects of *Puerarin* on the contents of adenosine nucleotides in *M. longissimus thoracis* of beef cattle under hot environment.

Item	Groups			*P*-value
	Control	Pue400	Pue800	
ATP (mg/g)	1.08 ± 0.06[a]	1.91 ± 0.04[b]	1.20 ± 0.22[a]	0.010
ADP (mg/g)	0.76 ± 0.04	0.84 ± 0.14	0.98 ± 0.04	0.288
AMP (mg/g)	2.13 ± 0.22	1.49 ± 0.02	1.87 ± 0.32	0.082
AMP/ATP	2.10 ± 0.13[a]	0.78 ± 0.03[b]	1.41 ± 0.29[b]	0.007

[a,b]Means within a row with no common superscript differ significantly ($P < 0.05$). All traits in this table were analyzed with cattle as the experimental unit ($n = 4$).

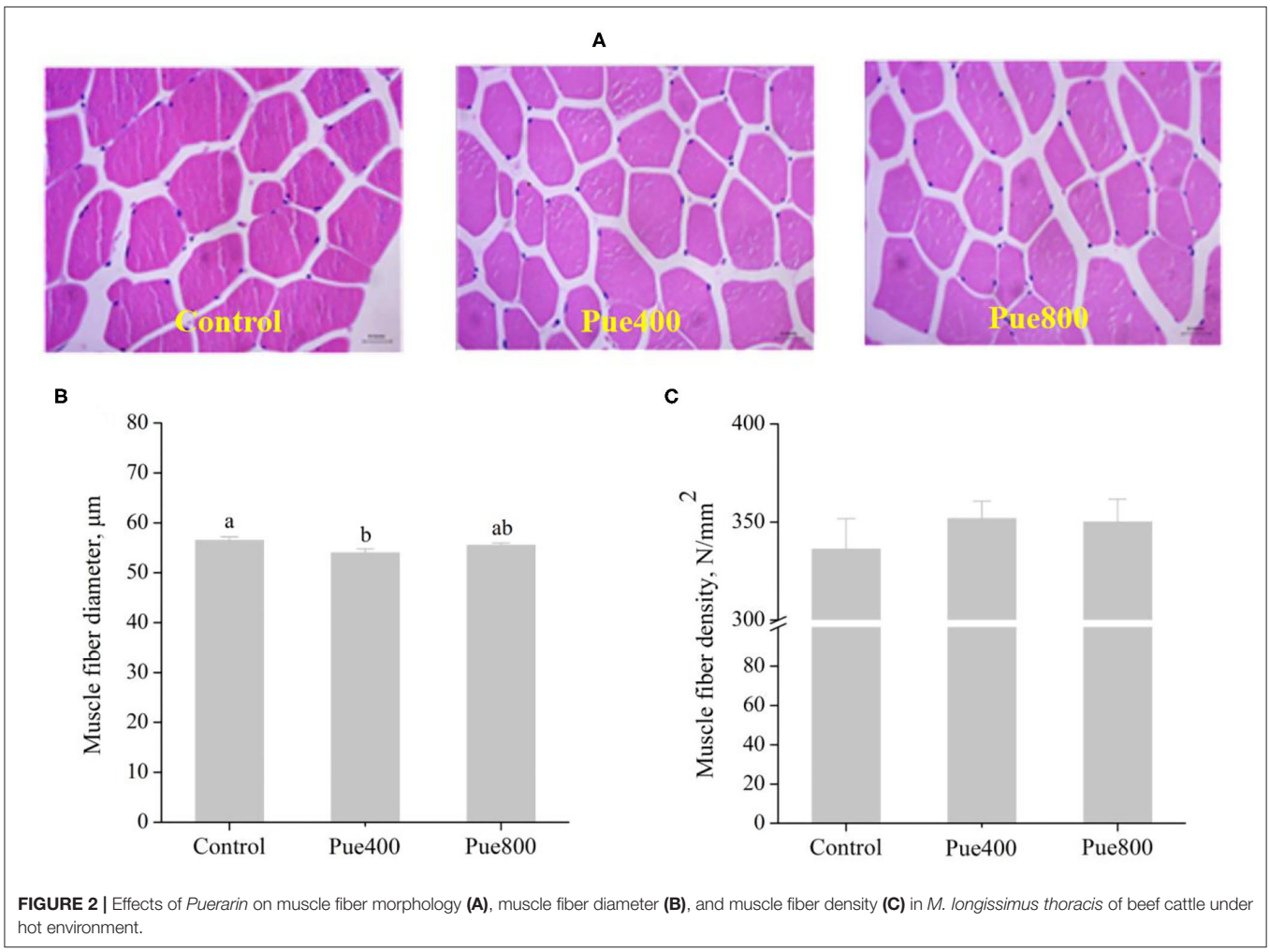

FIGURE 2 | Effects of *Puerarin* on muscle fiber morphology **(A)**, muscle fiber diameter **(B)**, and muscle fiber density **(C)** in *M. longissimus thoracis* of beef cattle under hot environment.

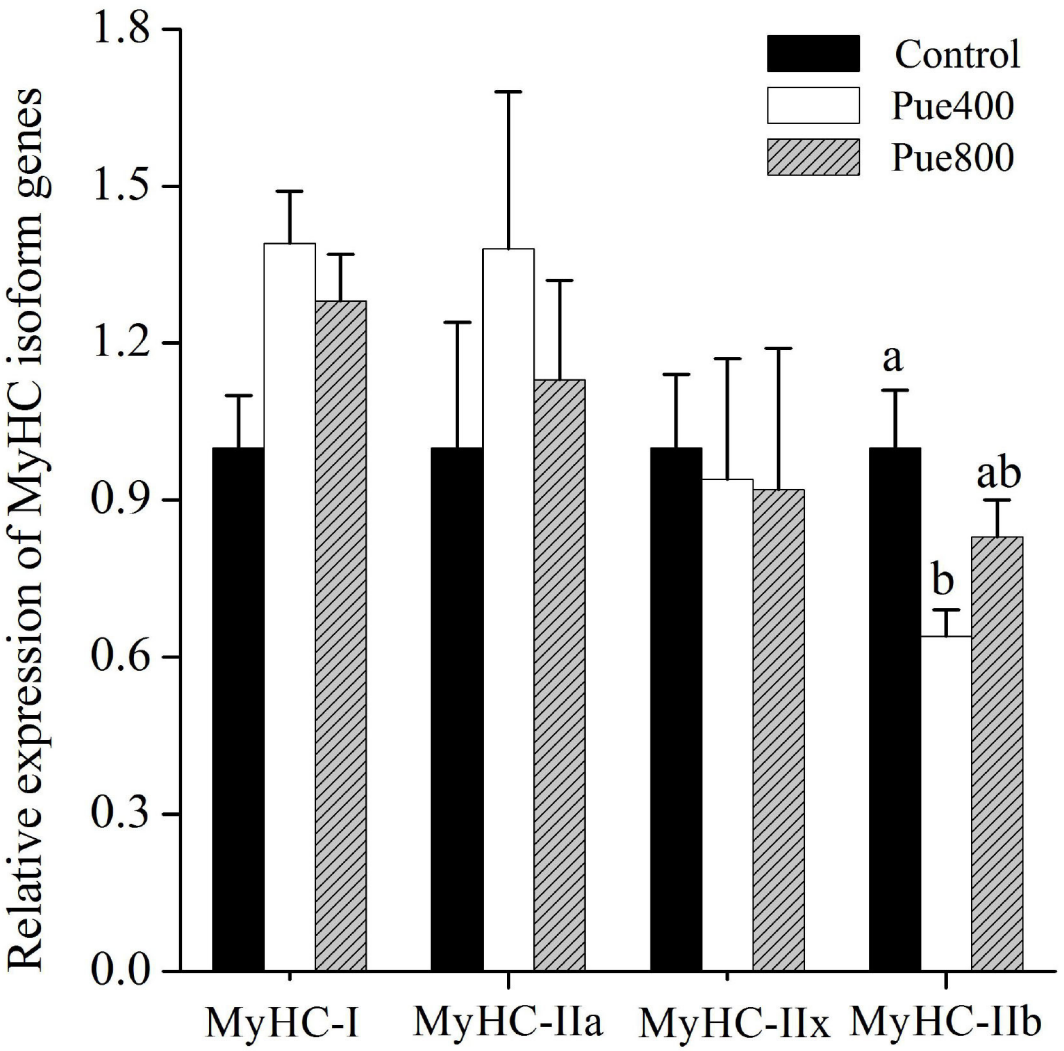

FIGURE 3 | Effects of *Puerarin* on the relative mRNA expressions of myosin heavy-chain (MyHC) isoform genes in *M. longissimus thoracis* of beef cattle under hot environment ($n = 4$). (■): dietary supplementation with 0 mg/kg *Puerarin* in the feed concentrate; (□): dietary supplementation with 400 mg/kg *Puerarin* in the feed concentrate; (▨): dietary supplementation with 800 mg/kg *Puerarin* in the feed concentrate.

rumen (25). There is evidence that *Puerarin* can be transformed into daidzein by bacterial enzymes in the large intestine of rats (26), and daidzein can be partly metabolized to equol by ruminal microorganisms (25). Daidzein and equol also have intrinsic antioxidant activities (27). Therefore, we speculated that *Puerarin* might have antioxidant effects for beef cattle in the form of daidzein, equol, and itself.

The tenderness of meat is the most important quality attribute influencing the consumer decision to purchase. There are complex interactions among various biochemical traits across multiple muscles affecting meat tenderness. One of the key events is the mitochondrial function. In skeletal muscle, mitochondria are abundant, and the power houses of cells function in muscle energy metabolism. Meanwhile, mitochondria are the main sites for ROS production; accumulation of damaged or dysfunctional mitochondria promotes an increase in ROS production (28). Mitochondrial functions are intrinsically linked to their morphology and membrane ultrastructure (29). Under physiological conditions, mitochondria are dynamic organelles that undergo permanent fission and fusion. While under heat stress conditions, the hypothalamic–pituitary–adrenal (HPA) axis was activated, which further induces calcium overload and promotes mitochondrial fission through phosphorylation of dynamin-related protein 1 (DRP1) (30). Our results were consistent with the theory that showed that heat stress disturbs the integrity of skeletal muscle cell membrane and keeps the most mitochondria in a fission state, while dietary supplementation with 400 mg/kg *Puerarin* promoted the integrity of cell membrane and maintained the mitochondrial morphology in round and oval shapes. Consistent with our results, Chen et al.

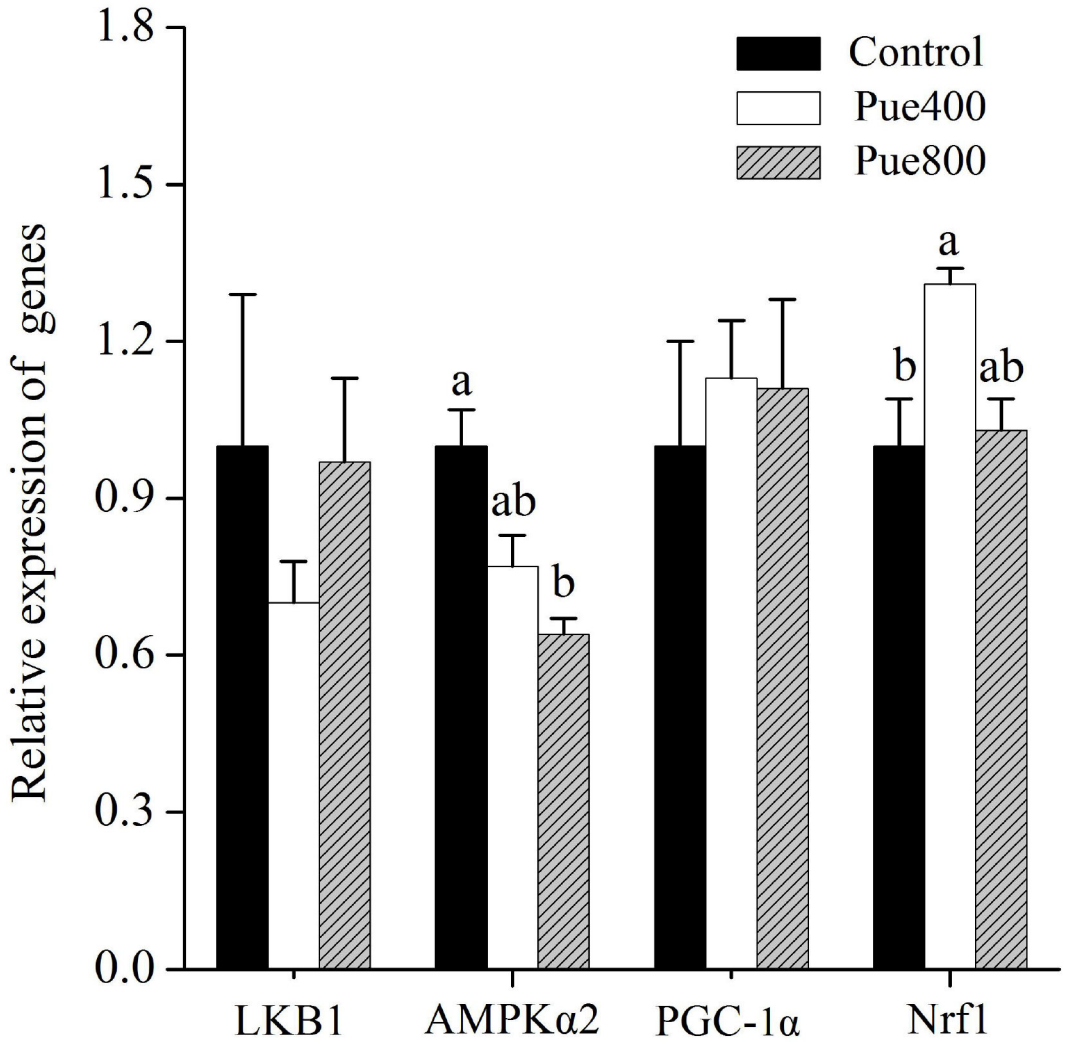

FIGURE 4 | Effects of *Puerarin* on the relative mRNA expression of LKB1, AMPKα2, PGC-1α, and Nrf1 in *M. longissimus thoracis* of beef cattle under hot environment ($n = 4$). (■): dietary supplementation with 0 mg/kg *Puerarin* in the feed concentrate; (□): dietary supplementation with 400 mg/kg *Puerarin* in the feed concentrate; (▨): dietary supplementation with 800 mg/kg *Puerarin* in the feed concentrate. LKB1, receptor serine/threonine kinase 1; AMPKα2, adenosine monophosphate-activated protein kinase α2; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; Nrf1, nuclear respiratory factor 1.

(31) reported that *Puerarin* could improve the function of mitochondria in the muscle of diabetic rats by upregulating mitochondrial biogenesis. These results suggested that *Puerarin* could attenuate oxidative stress induced by heat stress in beef cattle, which may help improve meat tenderness by inhibiting the oxidation of muscle proteins, enzymes, and lipid initiated by free radicals and protecting the structure and functional integrity of muscle mitochondria and enzymes (32). Consistently, our results showed that *Puerarin* supplementation decreased the shear force of the LT muscle compared with the control group.

Besides, long-term heat stress has been reported to increase the muscle fiber diameter and lead to a higher shear force of meat (33). Generally, the thinner and denser the muscle fiber, the more significant tenderness of the meat (34). Fortunately, in the current study, our results showed that dietary supplementation with *Puerarin* reduced the muscle fiber diameter of the LT muscle, which was also advantageous for increased tenderness. Many factors influence muscle fiber traits, among them, the muscle fiber type is one of the most pivotal factors with fine oxidative fibers and coarse glycolytic fiber (35). Previous studies reported that muscle with more slow-twitch type I fibers might improve tenderness in cattle (36), while an increasing proportion of MyHC-IIb fiber type in muscle usually lead to higher lightness and drip loss in raw meat (37). Correspondingly, in the present study, dietary supplementation with *Puerarin* decreased the mRNA expression of MyHC-IIb of the LT muscle in heat-stressed beef cattle. It has been accepted that heat stress stimulates the glycolysis process (38) and resulted in a decrease in slow muscle fiber composition (7). Therefore, we speculated that *Puerarin*'s modulation on muscle fiber type was dependent on

heat stress relief. However, further investigation is required to determine the specific regulation mechanism of muscle fiber type transformation induced by *Puerarin*.

Previous research showed that AMPK played an essential role in promoting muscle to assume a faster-contracting, more glycolytic nature (12). Recent studies reported that *Puerarin* alleviated autophagy by inhibiting the phosphorylation of AMPK (16). Similarly, our results showed that dietary supplementation with *Puerarin* decreased the mRNA expression of AMPK. The AMP/ATP ratio, as an essential regulator of AMPK activity (9), was also reduced by dietary supplementation with *Puerarin*. In the summer, oxidative stress induced by heat exposure leads to mitochondrial damage and impairing ATP synthesis (39), which ultimately results in a higher AMP/ATP ratio. Fortunately, Xue et al. (40) reported that *Puerarin* could stabilize mitochondrial potential by protecting the mitochondrial function. Correspondingly, in this study, the decreased AMP/ATP ratio in the LT muscle might correlate with the increased content of ATP induced by the benefit of *Puerarin* on mitochondrial function, whereas LKB1, which is also an upstream activator of AMPK, did not vary significantly among treatments.

PGC-1α, as a transcriptional activator, is shown to upregulate the slow fiber gene expression. While Nrf1 is the downstream target gene of PGC-1α, it has also been reported to involve the transformation of muscle fiber type (41). In this study, dietary supplementation with *Puerarin* numerically increased the mRNA expression of PGC-1α, whereas it significantly increased the Nrf1 gene expression. Similarly, in C2C12 cells, treatment with *Puerarin* increased the protein expression levels of PGC-1α and Nrf1 (42). Therefore, we speculated that *Puerarin* affected muscle fiber type of beef cattle via regulating AMPK and Nrf1 gene expressions under heat stress.

CONCLUSION

In conclusion, dietary supplementation with *Puerarin* could decrease the meat shear force of beef cattle under high temperature. Moreover, the improvement of meat quality was *via* improving muscle antioxidant ability and reducing the MyHC-IIb muscle fiber composition of the heat-stressed beef cattle. Based on the results of this study, we recommended 400 mg/kg *Puerarin* in the feed concentrate of beef cattle (∼300 kg) for mitigation of heat stress.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the study data are owned by Jiangxi Province Key Laboratory of Animal Nutrition. Access to these data would require additional approval beyond that of the authors. Requests to access these datasets should be directed to Dr. Yanjiao Li, yanjiaoli221@163.com.

ETHICS STATEMENT

This experiment was approved by the Committee for the Care and Use of Experimental Animals at Jiangxi Agricultural University (JXAULL-20190015).

AUTHOR CONTRIBUTIONS

YL, HS, and XS designed the overall study. HS, XZ, MQ, TP, BG, and YH performed the experiments. YL and XS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Impacts of Mootral on Methane Production, Rumen Fermentation, and Microbial Community in an *in vitro* Study

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Methane mitigation strategies have a two-sided benefit for both environment and efficient livestock production. This preliminary short-term *in vitro* trial using Mootral (garlic and citrus extracts), a novel natural feed supplement, was conducted to evaluate its efficacy on rumen fermentation characteristics, methane production, and the bacterial and archaeal community. The experiment was performed as a batch culture using rumen fluid collected from sheep, and Mootral was supplemented in three concentrations: 0% (Control), 10%, and 20% of the substrate (50% Grass:50% Concentrate). The rumen fermentation data and alpha diversity of microbial community were analyzed by ordinary one-way analysis of variance. The relative abundance and statistical significance of families and operational taxonomic units (OTUs) among the groups were compared by Kruskal–Wallis H test using Calypso software. After 24-h incubation at 39°C, Mootral in a dose-dependent manner improved the production of total volatile fatty acids and propionate while it reduced the acetate proportion and acetate/propionate ratio. The total produced gas was two times higher in the Mootral-supplemented groups than control ($P < 0.01$), while the proportion of methane in the produced gas was reduced by 22% ($P < 0.05$) and 54% ($P < 0.01$) for 10 and 20% Mootral, respectively. Mootral did not change pH, digestibility, and ammonia-nitrogen. Microbial community analyses showed that Mootral effectively changed the ruminal microbiome. The bacterial community showed an increase of the relative abundance of the propionate-producing family such as *Prevotellaceae* ($P = 0.014$) and *Veillonellaceae* ($P = 0.030$), while there was a decrease in the relative abundance of some hydrogen-producing bacteria by Mootral supplementation. In the archaeal community, *Methanobacteriaceae* was decreased by Mootral supplementation compared with control ($P = 0.032$), while the *Methanomassiliicoccaceae* family increased in a dose-dependent effect ($P = 0.038$).

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The results of the study showed the efficacy of the new mixture to alter the ruminal microbial community, produce more propionate, and reduce microbial groups associated with methane production, thus suggesting that Mootral is a promising natural mixture for methane reduction from ruminants.

Keywords: mootral, methane emission, rumen, bacteria, archaea

INTRODUCTION

Within livestock, ruminants are blamed to be the main contributors to greenhouse gas production, estimated approximately 80% of the total sector's emissions through enteric fermentation and about 14.5% of total anthropogenic greenhouse gas emissions (1). Enteric methane (CH_4) is produced by methanogenic archaea found mainly in the rumen, where they convert the hydrogen (H_2) and carbon dioxide (CO_2) produced from fermentation by a complex community of ciliate protozoa, bacteria, and anaerobic fungi to CH_4 (2). On the other hand, microbiome analysis has identified that numerous bacteria seem to be associated with variations in CH_4 production in ruminants (3). This enteric CH_4 emission is also associated with a dietary energy loss of 2–12%, hence reduced feed efficiency (4). Therefore, due to the negative environmental and animal production impacts, CH_4 mitigation has come forward in the last few decades (5).

To date, numerous efforts were made in order to reduce CH_4 emission from ruminants. These mitigation strategies include rumen manipulation, alteration of rumen fermentation, and modification of rumen microbial biodiversity by different means. Dietary manipulation is directly linked to changes in the rumen fermentation pattern and types of end products. A review highlighted that changing fermentation pattern is one of the most effective ways of CH_4 abatement (5). Researchers in the field of animal husbandry and nutrition are focusing on usage of natural products such as plant secondary metabolites (PSM) as environmentally safe alternatives to synthetic chemicals in ruminants' ration since the ban on the use of antibiotics and chemicals as a feed supplement in animals' feed (6). Researches have shown the ability of PSM to improve microbial activity and reduce CH_4 production through decreasing the number of ciliated protozoa and inhibiting methanogenic archaea (7–9).

Bioactive components extracted from garlic (*Allium sativum*), including several sulfur-containing compounds such as alliin, diallyl sulfides, and allicin, have been known for their antimicrobial efficacy (10) and studied to show their potentiality to reduce CH_4 production through direct inhibition of ruminal archaea (11). However, results showed some variations in CH_4 reduction capacity ranging from no reduction (12, 13) to 38.5% reduction (14). Besides, flavonoids are one of the important phytochemicals found in most citrus fruits and vegetables that have shown the potentiality to suppress CH_4 production (15). However, there is still rare information about flavonoid effects on rumen fermentation profile, with growing interest in the usage of flavonoids as natural feed supplements in ruminant feed (16).

Researchers are searching for new mixtures by combining many natural anti-methanogenic compounds that could be able to suppress CH_4 production effectively without impairing digestibility and volatile fatty acids (VFA) production (17). Accordingly, Mootral (Mootral SA, Rolle, Switzerland), a novel combination of garlic (*A. sativum*) powder and bitter orange (*Citrus aurantium*) extracts, has shown a great efficacy in reduction of CH_4 from ruminant through alteration of the archaeal community without impairing VFA production when used as a feed supplement in *in vitro* trial with 70% hay:30% concentrate diet using rumen fluid from cows (18).

However, there is still a limitation to find out its ability in different dietary regimen and in another ruminant species, as well as its impact on not only archaeal but also bacterial community in the rumen. Therefore, this preliminary small-scale *in vitro* study was performed using rumen fluid collected from sheep to evaluate the effect of Mootral supplemented to a 50% hay:50% concentrate diet on suppressing CH_4 production, alteration of ruminal microbiome (Bacterial and Archaeal), as well as rumen fermentation profile and digestibility.

MATERIALS AND METHODS

Rumen Fluid Collection

Rumen fluid was collected from three Corriedale wether sheep (body weight, 63.5 ± 4.4 kg) 4 h after the morning feeding using a stomach tube and vacuum pump. They were fed the basal diet of 50% concentrate and 50% Kleingrass (*Panicum coloratum*) hay at maintenance level for energy requirement. The first amount (100 ml) of the sucked rumen fluid was discarded to prevent contamination of saliva. The second amount was strained by four layers of absorbent gauze into an insulated container and transferred immediately to the laboratory. In the laboratory, the collected samples were mixed together in one beaker under a constant stream of CO_2 and kept in a water bath at 39°C prior to adding into the fermentation tubes. Animal management and sampling procedures were approved by the Animal Care and Use Committee of the Obihiro University of Agriculture and Veterinary Medicine (Approval Number 19–94).

Experimental Design and *in vitro* Incubation

The chemical composition of the feed used in the *in vitro* experiment is described in **Table 1**. The chemical composition of dry matter (930.15), organic matter (942.05), crude protein (984.13), and ether extract (920.39) was determined according to AOAC (19). Neutral detergent fiber, acid detergent fiber, and acid detergent lignin were measured and expressed inclusive of residual ash using an ANKOM^{200} Fiber Analyzer (Ankom Technology Methods 6, 5, and 8, respectively; ANKOM Technology Corp., Macedon, NY, USA). The neutral detergent

TABLE 1 | Chemical composition of the feed used in this study (g/kg dry matter).

(g/kg dry matter)	Concentrate	Kleingrass hay
Dry matter (in fresh matter)	837.9	849.6
Organic matter	942.0	916.2
Crude ash	58.0	83.8
Crude protein	229.3	135.8
Ether extract	40.1	38.1
Neutral detergent fiber	281.8	680.0
Acid detergent fiber	98.2	329.9
Acid detergent lignin	25.2	50.0

fiber was measured using sodium sulfite without heat-stable α-amylase. Three groups with three replicates for each were prepared: control (0% Mootral of substrate), 10% (10% Mootral of substrate), and 20% (20% Mootral of substrate). Each group is composed of 900 mg of ground feed (Concentrate + Kleingrass hay) as substrate added in a ratio of 50:50 in a sealed nylon bag (BG1020, Sanshin Industrial Co., Ltd., Kanagawa, Japan) that was placed in a 200-ml fermentation bottle. Feed was ground by a mill to pass through a 1-mm sieve. Mootral was used as a feed supplement added directly in the fermentation bottles in either 10% or 20% of the substrate. Mootral is composed of a mixture of nine parts garlic powder to one part citrus powder. The chemical composition of Mootral is described in **Table 2**. More information about formulation of Mootral has been published by Eger et al. (18). Thirty milliliters of rumen fluid and 60 ml of artificial saliva (20) were added in the fermentation bottles under continuous flushing with CO_2. Thereafter, tubes were sealed with rubber stoppers and aluminum caps. The incubation was performed for 24 h at 39°C. At the end of incubation, the produced headspace gas was measured using syringe. Culture fluid was used for measuring pH using pH meter (LAQUA F-72, HORIBA Scientific, Kyoto, Japan), and an aliquot was transferred into 1.5-ml tubes and centrifugated at 16,000×*g* at 4°C for 5 min. The supernatant and precipitation were collected and stored at −20°C.

In vitro Dry Matter Digestibility

Bags from each tube were washed by running tap water until the drain drops were clear. After that, they were dried in the oven at 60°C for 48 h to determine the *in vitro* dry matter digestibility (IVDMD) (21).

Gas Composition, VFA, and NH_3-N Analysis

The headspace gas samples were analyzed by injection of 1 ml of the gas using Hamilton gastight syringe (Hamilton Company, Reno, Nevada, USA) in a gas chromatograph (GC-8A, Shimadzu Corp., Kyoto, Japan) as described previously (22).

VFA were analyzed using high-pressure liquid chromatography (HPLC) (23). 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 (Shimadzu Corp., Kyoto, Japan) at 0.8 ml/min; conductivity detector (CDD-10AVP, Shimadzu Corp., Kyoto, Japan). Quantification of VFA concentration was performed using the external standard quantitation method.

For measuring the concentration of NH_3-N, samples were diluted 100 times using 0.1 M phosphate buffer (pH 5.5) and then analyzed according to the Modified Fujii-Okuda method (24) using NH_3 kit (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan).

DNA Extraction, Analysis of 16S Ribosomal RNA (16S rRNA), and Next-Generation Sequencing

DNA was extracted from rumen fluid samples using repeated beads beating plus column (RBB+C) method and the Maxwell 16 LEV blood DNA kit (Promega, Madison, WI, USA) (25, 26). The concentration and purity of extracted DNA were measured by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Tokyo, Japan), and then the DNA concentration was adjusted to 5 ng/μl using Tris-EDTA buffer. The variable regions (V3 and V4) of bacterial 16S rRNA gene were amplified from the purified DNA. The primers used in this study consisted of the Illumina overhang adapters and universal primers in the first stage of PCR as follows: the forward overhang adapter and bacterial universal primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and the reverse overhang adapter and bacterial universal primer 5′-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATTACHVGGGTATCTAATCC-3′. For Archaeal 16S rRNA gene, the forward overhang adapter and Arch349F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGYGCASCAGKCGMGAAW-3′) and the reverse overhang adapter and Arch806R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACVSGGGTATCTAAT-3′) were used. In the second PCR, Illumina sequencing adapters and dual index barcodes were added to the amplicons using Nextera® XT Index Kit (Illumina Inc., San Diego, California, USA). The concentration of the second PCR product was quantified using a Quantus™ fluorometer (QuantiFluor® dsDNA System, Promega, Madison, Wisconsin, USA) and then PCR products from all samples were pooled in one tube in equal amounts. Paired-end sequencing was performed using the Illumina MiSeq (Illumina, San Diego, California, USA). The preparation of 16S rRNA gene amplicon was done as previously described by Pelpolage et al. (27).

The analysis of raw 16S rRNA gene sequence was done according to the method described before by Warren et al. (25). Samples with <1,000 sequence reads were removed. Sequence reads were clustered into operational taxonomic units (OTUs) with a 97% sequence identity threshold. The generated biome table was used in Calypso version 8.84 to generate a principal coordinate analysis (PCoA) 3D plot and express relative abundance of bacterial or archaeal taxa among experimental groups. For the archaeal community, samples were rarefied to a read depth of 1,678, while in the bacterial community, there were 16,246 reads. Nucleotide sequence data reported in this study are

TABLE 2 | Chemical composition of Mootral powder (g/100 g dry matter).

(g/100 g dry matter)	Mootral
Crude ash	3.9
Crude protein	22.0
Crude fat	0.51
Crude fiber	1.9
Sodium	0.04

available in the DDBJ Sequence Read Archive under the accession numbers DRA011192.

Statistical Analysis

All rumen fermentation profile data and alpha diversity of both the bacterial and archaeal community were analyzed statistically using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, California, USA). Data were provided as means ± SEM (standard error of the mean). Ordinary one-way analysis of variance (ANOVA) was performed followed by Tukey's test to find the significance among experimental groups. The relative abundance and statistical significance of families and OTU among the three experimental groups were compared using Kruskal–Wallis H test in Calypso version 8.84. Differences were considered statistically significant when *P*-value was <0.05, and tendency was declared when *P*-value was between 0.05 and 0.10.

RESULTS

Effect of Mootral on Rumen Fermentation Characteristics

Mootral exhibited significant changes in the rumen fermentation profile as shown in **Table 3**. However, pH, IVDMD, and NH_3-N did not show any differences ($P > 0.05$) among experimental groups. The total gas produced was increased ($P < 0.01$) for Mootral-supplemented groups compared with control. In contrast, the percentage of the volume of CH_4 in the produced gas was decreased by increasing Mootral dose; it showed a reduction of up to 21.98% for 10% Mootral ($P < 0.05$) and 54.25% for 20% Mootral ($P < 0.01$) when compared with control (**Figure 1A**). However, the volume of the produced CH_4 per day and CH_4/DM were not different between Mootral-treated groups and control ($P > 0.05$), but there was an increase in 10% compared to 20% ($P < 0.05$) (**Table 3**). The percentage of the volume of CO_2 in the produced gas was increased by increasing Mootral supplementation. It was higher for 20% when compared with control ($P < 0.01$) and with 10% ($P < 0.05$), and it was also higher for 10% compared with the control group ($P < 0.05$) (**Figure 1B**). Consequently, the total amount of CO_2 produced in 24 h was higher ($P < 0.01$) by increasing Mootral dosages (69.54 and 76.51 ml for 10% and 20%, respectively) when compared with control (35.01 ml) (**Table 3**). The CH_4/CO_2 ratio in the total produced gas decreased with increasing Mootral dosage ($P < 0.01$) (**Table 3**).

Acetic acid ratio showed a reduction among groups ($P < 0.01$) with increasing Mootral supplementation. However, the propionic acid concentration and ratio increased with increasing Mootral supplementation with a significant effect between 20% and control ($P < 0.01$) and between 20% and 10% ($P < 0.05$). Mootral did not affect either the concentration or percentage of butyric acid among groups. The total VFA increased with Mootral supplementation where it reached a significant level between 20% and the control group ($P < 0.05$). The acetate/propionate (A/P) ratio decreased with increasing Mootral dosage ($P < 0.01$) (**Table 3**).

Effect of Mootral on Bacterial Community Diversity and Composition

The minimum and maximum sequence reads were 16,246 and 42,030, respectively. The α-diversity indices including Richness, Choa1, Evenness, and Shannon index were not affected by Mootral supplementation ($P > 0.05$) (**Table 4**). However, based on β-diversity analysis at the family level, the control samples were clustered away from Mootral-supplemented groups. Also, Mootral treated samples were clustered close to each other (**Figure 2A**).

Based on family level, Mootral was able to change the bacterial composition by increasing the relative abundance of family *Prevotellaceae* especially in 20% Mootral (28.07%); this increase was significant when compared with the control group (23.16%) ($P < 0.05$) and with 10% Mootral (23.32%) ($P < 0.05$), while there was no difference between 10% Mootral and control ($P = 0.99$) (**Figure 3A**). Similarly, the relative abundance of the family *Veillonellaceae* increased ($P < 0.05$) in 20% Mootral (7.98%) compared with control (6.62%), while the abundance of *Veillonellaceae* in 10% Mootral (7.1%) was not statistically different from that in control ($P = 0.46$) (**Figure 3B**). Based on OTU level, some bacterial strain belonging to family *Ruminococcaceae* and order *Bacteroidales* significantly decreased between Mootral-supplemented groups and control ($P < 0.05$) (**Supplementary Figure 1**). The relative abundance of all taxa at the phylum and family level of bacteria, which was more than 0.1% of total bacteria, was described in **Supplementary Table 1**.

Effect of Mootral on Archaeal Community Diversity and Composition

The minimum sample sequence read was 1678 and the maximum was 19,986. Mootral supplementation did not alter the α-diversity indices of the archaeal community among experimental groups (**Table 4**). However, the β-diversity analysis at the family level showed a shift of the control group samples to be away from Mootral-supplemented samples (**Figure 2B**).

Mootral-supplemented groups showed a significant shift in the archaeal community by decreasing the relative abundance of the major methanogenic group, family *Methanobacteriaceae* (94.07 and 92.70% for 10 and 20% Mootral, respectively), when compared with the control group (96.42%) ($P < 0.05$) (**Figure 4A**). In contrast, the relative abundance of family *Methanomassiliicoccaceae* increased with increasing Mootral dosages (5.83 and 7.12% for 10 and 20% Mootral, respectively) when compared with non-supplemented group (3.5%) ($P < 0.05$) (**Figure 4B**). The relative abundance of all taxa at the phylum and family level of archaea that was more than 0.1% of total archaea was described in **Supplementary Table 1**.

TABLE 3 | Rumen fermentation characteristics of 24-h *in vitro* incubation.

Parameter	Experimental groups			*P*-value
	0%	10%	20%	
pH	6.87 ± 0.04	6.86 ± 0.01	6.80 ± 0.01	0.13
Total gas production (ml)	43.43 ± 1.29^{b}	81.93 ± 2.56^{a}	84.03 ± 2.32^{a}	<0.01
IVDMD[1] (%)	62.70 ± 4.70	59.10 ± 1.40	59.00 ± 3.30	0.70
CH_4 (ml)	8.42 ± 0.41^{ab}	12.39 ± 0.52^{a}	7.52 ± 1.48^{b}	0.02
CH_4/DM (ml/g)	11.12 ± 0.54^{ab}	16.39 ± 0.68^{a}	9.89 ± 1.95^{b}	0.02
CO_2 (ml)	35.01 ± 1.01^{c}	69.54 ± 2.12^{b}	76.51 ± 0.87^{a}	<0.01
CH_4/CO_2 ratio	0.241 ± 0.01^{a}	0.178 ± 0.00^{b}	0.098 ± 0.02^{c}	<0.01
Acetate (mmol/L)	48.22 ± 1.70	48.13 ± 0.26	48.85 ± 0.33	0.86
Propionate (mmol/L)	21.77 ± 1.21^{b}	24.83 ± 0.28^{b}	30.28 ± 0.94^{a}	<0.01
Butyrate (mmol/L)	11.56 ± 0.86	12.33 ± 0.24	13.03 ± 1.24	0.54
Total VFA[2] (mmol/L)	81.55 ± 3.64^{b}	85.29 ± 0.73^{ab}	92.16 ± 0.68^{a}	0.04
Acetate (%)	59.18 ± 0.79^{a}	56.44 ± 0.27^{b}	53.01 ± 0.08^{c}	<0.01
Propionate (%)	26.67 ± 0.60^{b}	29.11 ± 0.12^{b}	32.87 ± 1.24^{a}	<0.01
Butyrate (%)	14.14 ± 0.43	14.45 ± 0.17	14.12 ± 1.26	0.95
A/P ratio[3]	2.22 ± 0.08^{a}	1.94 ± 0.02^{b}	1.62 ± 0.06^{c}	<0.01
NH_3-N (mg/dl)	11.07 ± 1.38	11.51 ± 0.84	10.33 ± 0.20	0.69

^{a,b,c}Values within the same row with different superscripts are different ($P < 0.05$). [1]IVDMD, in vitro dry matter digestibility; [2]VFA, volatile fatty acids; [3]A/P, acetate/propionate ratio. ±, standard error of the mean.

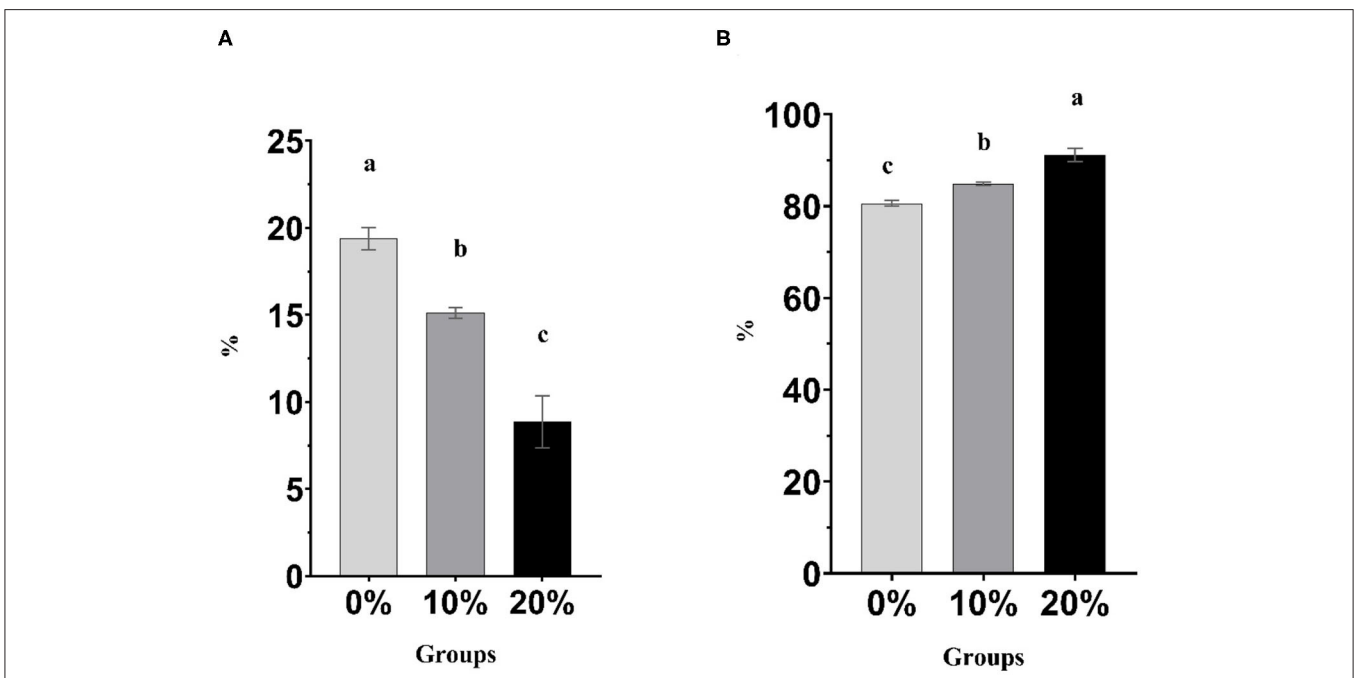

FIGURE 1 | (A) Percentage of CH_4 in the produced gas. **(B)** Percentage of CO_2 in the produced gas. Bars with different superscripts are different ($P < 0.05$).

DISCUSSION

Previous studies have shown the efficacy of garlic compounds and flavonoids on CH_4 suppressing of either *in vivo* or *in vitro* studies (15, 28, 29); however, there are still variations on VFA productions and digestibility (15, 30–32). Combination of these two natural products may have a significant effect on reducing CH_4 without impairing rumen fermentation characteristics. To our knowledge, only two studies were performed using that combination of garlic and citrus (Mootral) to evaluate its effect on CH_4 emission (33) and fermentation profile through alterations of the archaeal community (18), while the bacterial community

TABLE 4 | α-Diversity of microbial community based on OTU level.

Parameter	Experimental groups			*P*-value
	0%	10%	20%	
Bacteria[a]				
Richness	992.60 ± 7.63	1004.00 ± 5.27	973.60 ± 9.59	0.08
Chao1	1035.00 ± 0.73	1043.00 ± 0.12	1034.00 ± 4.94	0.14
Evenness	0.825 ± 0.01	0.840 ± 0.01	0.823 ± 0.01	0.17
Shannon index	5.72 ± 0.06	5.84 ± 0.04	5.69 ± 0.05	0.14
Archaea[b]				
Richness	226.70 ± 20.26	185.90 ± 28.40	232.60 ± 13.37	0.32
Chao1	408.70 ± 7.20	326.70 ± 60.54	406.70 ± 8.60	0.25
Evenness	0.742 ± 0.24	0.731 ± 0.02	0.750 ± 0.01	0.81
Shannon index	4.43 ± 0.17	4.09 ± 0.26	4.45 ± 0.09	0.33

[a]*α-Diversity of bacteria of the experimental groups. Samples rarefied to a read depth of 16,246.* [b]*α-Diversity of archaea of the experimental groups. Samples rarefied to a read depth of 1,678.* ±*, standard error of the mean.*

has not been investigated yet. Further investigations are required to ensure efficacy in different diet forms and different ruminant species. Therefore, this preliminary *in vitro* trial was performed to evaluate that efficacy in a different feeding regimen (50% Kleingrass hay:50% Concentrate) and another ruminant species (sheep) through studying its impacts on the rumen fermentation profile and archaeal and bacterial communities.

Mootral Improved Rumen Fermentation Characteristics by Reducing the Percentage of Methane

Most of the anti-methanogenic products studied before showed negative effects on fermentation profile at high doses to achieve the effective CH_4 reduction (11, 17, 34). However, the results of the current study showed an increase in propionic acid and total VFA with increasing Mootral dosages that may be due to stimulation of the family *Prevotellaceae* that increased in the current study. *Prevotellaceae* is well-known as a propionate-producing bacteria (35). Many researches have proved that fermentation leading to more propionate is strongly associated with decrease in CH_4 production. For instance, Ungerfeld (36) reported that reduction of CH_4 in batch cultures leads to redirection of metabolic H_2 toward propionate production. Similarly, Kittelmann et al. (37) assumed that high propionate was present in low-CH_4-emitting cows. The improvement in the production of total VFA by Mootral effect was also observed in a previous study (18) and in other studies that used either garlic (38) or flavonoids (39). The reduction of acetate and A/P ratios was also reported with the inclusion of 300 mg/L garlic oil in a continuous culture system (40) and garlic powder (30). Production of more propionate and less acetate means that H_2 was redirected toward propionate formation as an alternative way other than methanogenesis (41). The rumen medium was stabilized in the presence of garlic and citrus and did not change the pH, IVDMD, and NH_3-N as shown previously (39, 42, 43) and similarly when the same mixture was used (18).

Mootral supplementation showed a strong efficacy to reduce the CH_4 percentage in the produced gas up to 54%. Interestingly, the total gas produced (ml/day) was two-fold more in Mootral-supplemented groups than control and that explains why the total CH_4 production/day was not changed between Mootral groups and the control. The trial data of an *in vitro* gas production monitoring for 48 h from Copenhagen University (44) showed a similar finding to our results. Mootral stimulated fermentation, and the total gas production increased during the first 16 h. Also, they reported that Mootral reduced the percentage of CH_4 in the produced gas with 58% from a typical Danish dairy feed ration without affecting IVDMD. The substantial increase in total gas production and CO_2 with reducing CH_4/CO_2 ratio as an important indicator of rumen fermentation profile in the current study might be due to the stimulating effect of Mootral on the activity of some rumen microbes other than methanogens. This interesting finding has to be proven in further researches using qPCR. Eger et al. (18) reported that Mootral reduced the percentage and the total production of CH_4 in the RUSITEC system as a long-term study (14 and 18 days). As our present study was a batch culture for short term (24 h), these differences could contribute to the result's discrepancy.

Mootral Changed the Bacterial Community Composition

Although Mootral was effective in shifting the bacterial community toward less H_2-producing bacteria, the α-diversity was not different. By analyzing the bacterial community of ruminants, it has been proven that differences in the bacterial community composition were associated with the level of CH_4 emissions (37). The results of the current study revealed that *Prevotellaceae*, the main dominant family in rumen fluid (45), was higher with Mootral groups especially the higher dose (20%) as mentioned previously. The genus *Prevotella* is well-known to produce propionate by utilizing H_2 *via* the randomizing (succinate) or non-randomizing (acrylate) pathways through the fermentation of carbohydrates. These pathways were the main ways for consumption of H_2, which accumulated as a

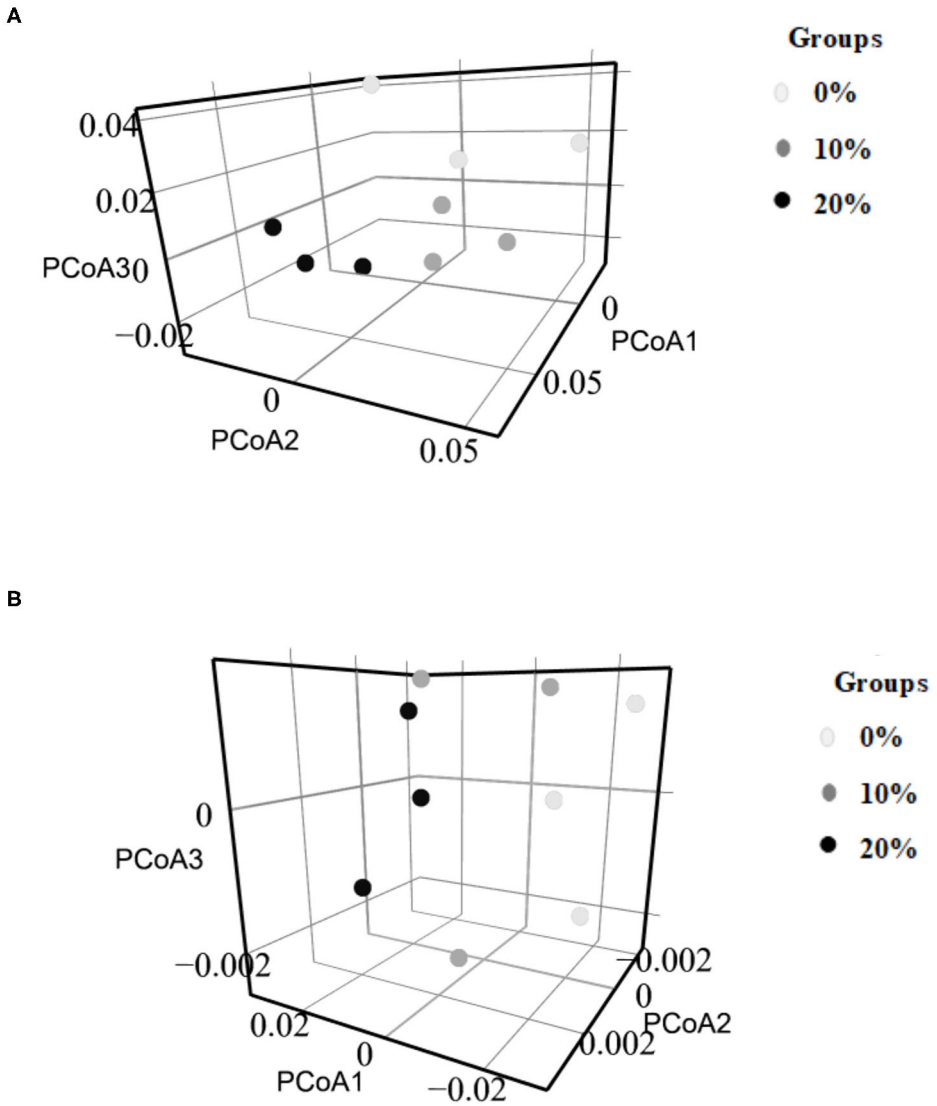

FIGURE 2 | Beta diversity of microbial community analysis by PCoA 3D. **(A)** Bacterial community (Family level), **(B)** Archaeal community (Family level).

consequence of reduced methanogenesis (35). Similarly, the family *Veillonellaceae* showed a higher abundance in Mootral groups. This might be due to the effect of flavonoids extracted from citrus (15). *Megasphaera elsdenii* belonging to the family *Veillonellaceae* is well-known as lactate-utilizing and propionate-producing bacteria (46). Moreover, *Quinella* spp., a member of the family *Veillonellaceae*, were more numerous in low-CH_4-producing cows (47). Tapio et al. (3) also reported that the lower CH_4 production ruminotype possessed a high relative number of propionate-producing *Quinella ovalis* and succinate-producing bacteria such as *Prevotella bryantii*.

Within bacteria, some species belonging to *Ruminococcaceae, Clostridiales,* and *Bacteroidales* are H_2 producers, while *Prevotella* spp. are net H_2 utilizers (48). Denman et al. (35) attributed that CH_4 emission depends on the abundance of H_2-producing and -consuming bacteria. The results from the current study based on OTU level showed a lower abundance of some OTUs belonging to *Ruminococcaceae and Bacteroidales*, while some OTUs belonging to genus *Prevotella* were higher in Mootral-treated groups compared to the control group. Similar findings were observed by Popova et al. (49) who found that reduction of CH_4 by linseed and nitrate reduced the relative abundance of *Ruminococcaceae* as well, and linseed supplementation increased the proportion of *Prevotellaceae.* The combination effect of Mootral on the bacterial community is still unclear, but it could have an indirect effect on increasing the relative abundance of H_2-utilizing bacteria by reducing methanogenesis and stimulating the utilization of accumulated H_2 by those bacteria to produce propionate. There is a need to further understand the mode of action of Mootral to these bacteria in upcoming researches.

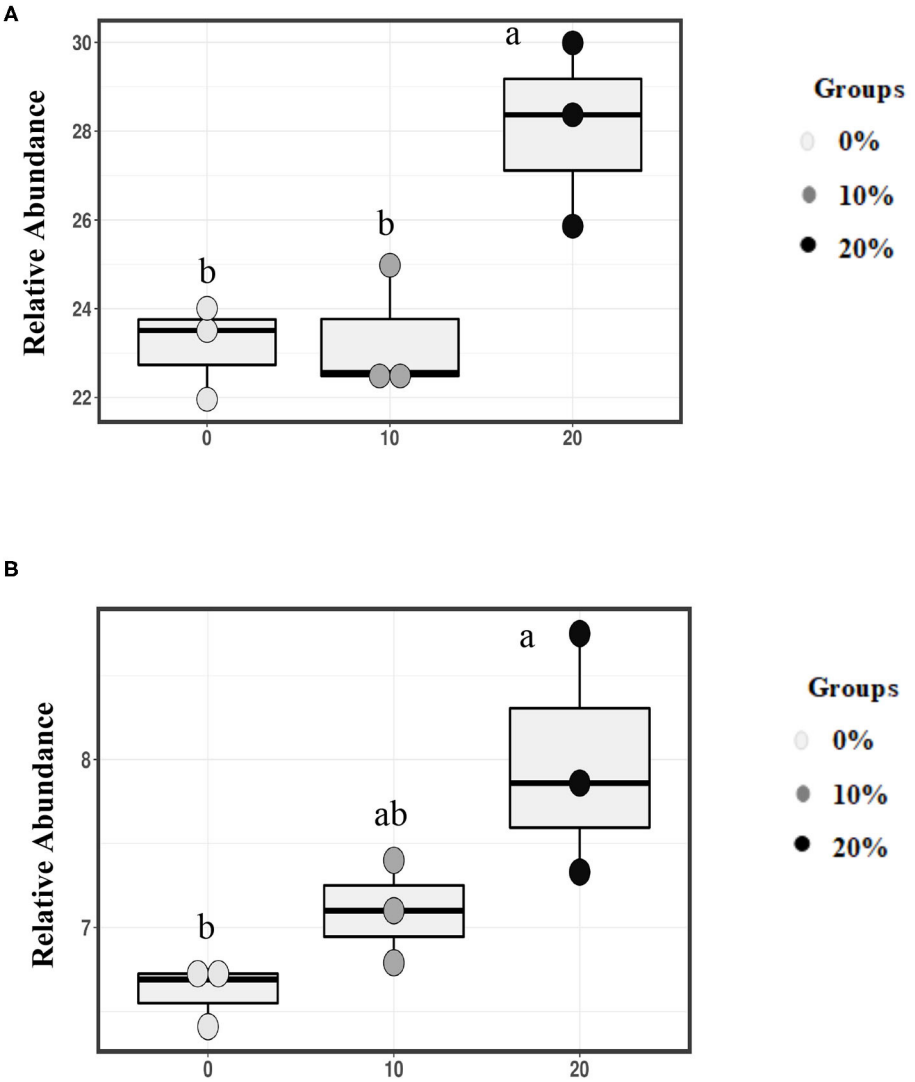

FIGURE 3 | Relative abundance of families. **(A)** *Prevotellaceae*, and **(B)** *Veillonellaceae* in total bacterial community. Boxes with different superscripts are different ($P < 0.05$).

Mootral Altered the Archaeal Community Composition

Similar to the results in the bacterial community, the archaeal α-diversity did not show any changes with Mootral supplementation, which was similar to previous findings (18). The archaeal sequences were assigned to two dominant families, *Methanobacteriaceae* and *Methanomassiliicoccaceae*. These two families were also dominant in other 16S rRNA gene-based studies (50–52). Mootral showed significant changes in the archaeal community by decreasing the relative abundance of the dominant family group *Methanobacteriaceae*. The family *Methanobacteriaceae* includes the genus *Methanobrevibacter*, which is a well-known major CH_4 producer in the rumen (53, 54). The reduction of the family *Methanobacteriaceae* might be related to the direct effect of organosulfur compounds of garlic in Mootral through interaction with cell membrane and inhibiting certain SH-containing enzymes essential for metabolic activities of methanogenic archaea (11). The effect of garlic on reduction of archaea has also been shown in previous studies (11, 55). Furthermore, it has been reported that flavonoids may have an effect on methanogenic archaea populations (43). Additionally, Oskoueian et al. (15) reported that flavonoids such as naringin and quercetin at the concentration of 4.5% of the substrate suppressed CH_4 production through reduction of total methanogens. However, researchers are still not aware of the mode of action of flavonoids on archaea. Ruminal ciliated protozoa could enhance the methanogenesis as they are a major H_2 producer in the rumen, and they act as a host for methanogens. The produced H_2 is utilized by archaea found either inside or on the external surface of the protozoal cells

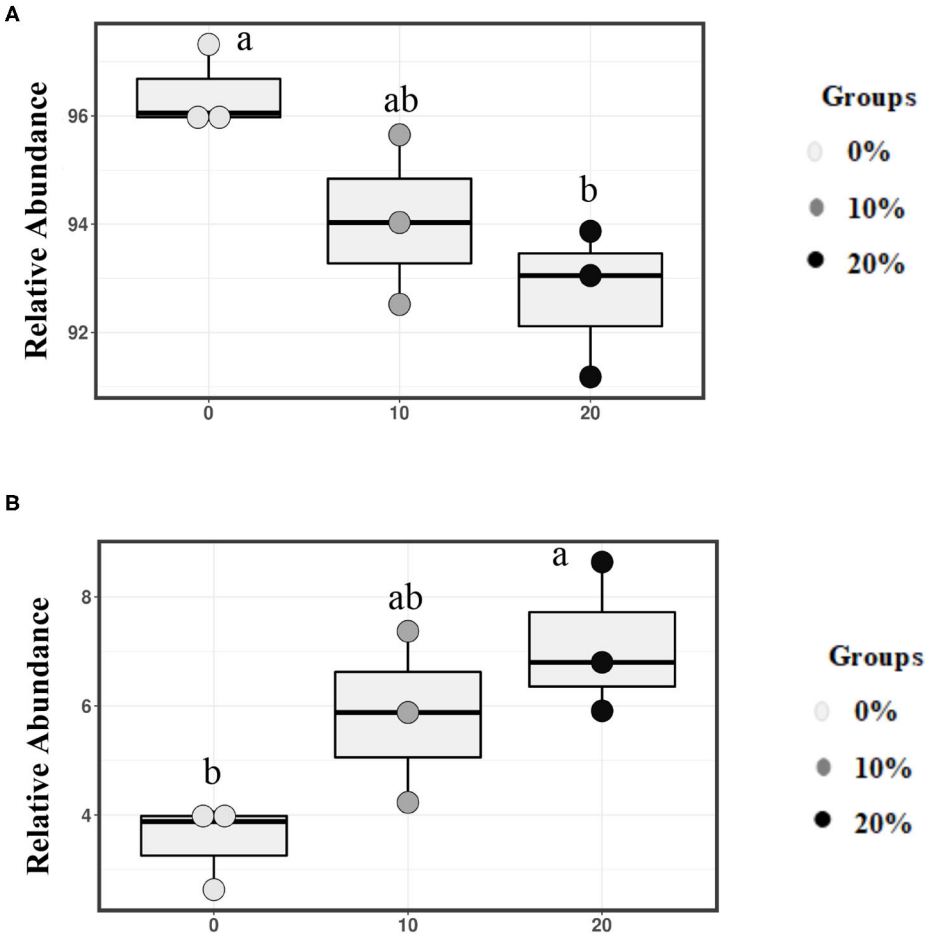

FIGURE 4 | Relative abundance of families. **(A)** *Methanobacteriaceae*, and **(B)** *Methanomasillicocae* in total archaeal community. Boxes with different superscripts are different ($P < 0.05$).

(56). *Methanobacteriaceae* with its species were found to be associated with protozoa (57, 58). It has been shown that flavonoid supplementation reduced the total protozoal number (15), which has not been investigated in the current study. Thus, further researches are required to study the effect of Mootral on protozoa.

Interestingly, the family *Methanomassiliicoccaceae* showed a higher abundance in Mootral groups in a dose-dependent pattern. St-Pierre and Wright (59) reported that its normal abundance within archaea in the rumen is about 5%, which was similar to the results of the current study. To date, as a new archaeal group, information on this taxonomy remains limited (60). A comprehensive understanding of the community of the family could help to know its function in the rumen. However, Danielsson et al. (61) also reported that *Methanomassiliicoccaceae* was 1.5-fold more abundant in low CH_4 emitters than in high CH_4 emitters. Moreover, its abundance was higher in a microbial community with low CH_4 production such as cows supplemented with nitrate (62) as well as in a previous *in vitro* Mootral study (18).

CONCLUSION

Mootral supplementation reduced the CH_4 percentage and CH_4/CO_2 ratio in a dose-dependent manner. Mootral was able to shift the fermentation to produce more propionate and less acetate and to increase the production of total VFA without affecting IVDMD. Furthermore, 20% Mootral was effective in increasing the abundance of H_2-consuming groups such as *Prevotellaceae* and *Veillonellaceae* and in reducing some H_2-producing bacteria. In addition, the archaeal community was altered by reducing the major CH_4-producing family *Methanobacteriaceae* and increasing *Methanomassiliicoccaceae*. The results of this study suggest that Mootral as a new combination could have the potentiality to be used for reduction of CH_4 in ruminants.

DATA AVAILABILITY STATEMENT

Nucleotide sequence data reported in this study are available in the DDBJ Sequence Read Archive under the accession number DRA011192.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine.

AUTHOR CONTRIBUTIONS

EA, DK, TN, and NF: conceptualization. EA, DK, and NF: methodology. MH and TN: validation. EA, RY, and MF: formal analysis and investigation. NF and DK: resources. EA: writing—original draft preparation. RY, MF, DK, MH, TN, and NF: writing—review and editing. EA: visualization. MH, TN, and NF: supervision. NF: project administration. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.623817/full#supplementary-material

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Influence of a Polyherbal Mixture in Dairy Calves: Growth Performance and Gene Expression

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A polyherbal feed mixture containing (*Achyrantes aspera*, *Trachyspermum ammi*, *Citrullus colocynthis*, *Andrographis paniculata*, and *Azadirachta indica*) was evaluated in growing calves through blood chemistry, blood biometry, and gene expression during the pre-ruminant to weaning period. Forty Holstein calves (initial BW 45.6 ± 3.2 kg; 22.8 ± 0.9 days post birth) from a dairy farm were randomly assigned to the following treatments: 0, 3, 4, and 5 g/d of a polyherbal mixture, dosed in colloid gels with gelatin. Calves were housed in individual outdoor boxes with *ad libitum* access to a 21.5% CP calf starter and water and fed individually with a mixture of milk and a non-medicated milk replacer (22% CP). Blood samples were collected on day 59 for blood chemistry, blood biometry, and gene expression analysis in leukocyte through microarray assays. Immunoglobulins were quantified by enzyme-linked immunosorbent assay. The animals treated with the polyherbal mixture showed a quadratic effect on final body weight, daily weight gain, final hip height, and final thoracic girth. The best performance results were obtained with a treatment dose of 4 g/d. The serum IgG increased linearly with the treatment doses. Gene set enrichment analysis of upregulated genes revealed that the three biological processes with higher fold change were tight junction, mucin type O-Glycan biosynthesis, and intestinal immune network for IgA production. Also, these upregulated genes influenced arachidonic acid metabolism, and pantothenate and CoA biosynthesis. Gene ontology enrichment analysis indicated that the pathways enriched were PELP1 estrogen receptor interacting protein pathways, nuclear receptors in lipid metabolism and toxicity, tight junction, ECM-receptor interaction, thyroid hormone signaling pathways, vascular smooth muscle contraction, ribosome function, glutamatergic synapse pathway, focal adhesion, Hippo, calcium, and MAPK signaling pathways.

Keywords: growth performance, Holstein calves, animal nutrition, nutrigenomics, feed plant additive

INTRODUCTION

In dairy farms, respiratory and enteric diseases can occur, causing a high rate of mortality in young animals, significant economic losses (1, 2), and lower resistance to caloric stress (3, 4). In these animals, the feed intake is affected, which seriously compromises the development of the immune system (5). These diseases require the use of antibiotics which have been banned as growth promotors in several countries (6). There is an increase in public pressure for the usage of these drugs in animals, and therefore, the evaluation of feed plant additives with beneficial effects in adult ruminants on indicators of health and production are critical for raising calves (7–9).

In adult ruminants, a polyherbal mixture with phosphatidylcholine (PCho) and other nutraceutical metabolites results in improved performance, and health suggesting it may have beneficial effects on calves (10–12). Dietary PCho stimulates the IL-2 formation in the spleen and CD25, CD28, CD71 expression (13), improving the immune response. Choline is part of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet-activating factor), a recognized activator of immune response (14, 15), and rumen-protected choline can improve the immune response and health in cows (16–18); however, evaluations in calves before weaning are missing.

The objective of this study was to evaluate the effect of an herbal product, with PCho elaborated with medicinal plants from India (*Achyrantes aspera*, *Trachyspermum ammi*, *Citrullus colocynthis*, *Andrographis paniculata*, and *Azadirachta indica*), on the productive performance and immune response in Holstein calves. These plants have already been evaluated by our researching group (10–12) and the results obtained showed beneficial effects on production in adult animals. However, the activity of these plants on young animals is unknown.

MATERIALS AND METHODS

Animals, Diet, and Experimental Design

The experimental procedures were performed in accordance with the recommendations of the CIOMS (19), observing the standards for ethics, biosafety, and animal well-being approved by the institutional committee. The experiment was conducted in a commercial farm located in the central part of the northern region of México (25°39' 14.4 "N 103°27' 27.8" W; elevation 1,139 m). The climate was semi-dry, with extreme temperatures, with an annual rainfall of 250.6 mm. Average temperature was 20.21°C, with a maximum of 33.60°C and a minimum of 5.59°C. Forty Holstein calves (initial BW 45.6 ± 3.2 kg) received individual doses of a polyherbal mixture (BioCholine, Indian Herbs). The doses were defined based on metabolic weight using results from sheep experiments (10, 12) and were as follows: 0, 3, 4, and 5 grams per day (g/d). The herbal products were prepared in colloid gels with gelatin to ensure individual daily consumption in the morning during the 90 days that the experiment lasted. The polyherbal-gelatin mixture (PGM) was prepared 1 day before administration. Calves were clinically healthy at the beginning of the experiment and had an age of 22.8 ± 0.9 days post birth. Calves were weighted and measured at the beginning and at the end of the experiment (90 days of evaluation). During weighing, the hip and wither heights as well as the thoracic girth were measured (20). All calves received colostrum following birth, and immunoglobulins in the blood were measured by refractometry.

Calves were individually placed in outdoor boxes (2.00 × 1.25 m), with water *ad libitum,* and fed twice a day (7.00 am and 17.00 pm). They received a mixture of milk (56%) and a non-medicated milk replacer (44% milk replacer Nu-3; Grupo Nu-3 balanced food, Guanajuato, México) with 4% moisture, 22% crude protein, 15% fat, 0.1% crude fiber, 6.0% ash, and 52.9% nitrogen-free extract (NFE), prepared with 130 g/L water. The milk powder (143 g/L) was reconstituted in hot water (65°C) and fed at a temperature of 39°C in a container; prior to feeding, it was mixed with milk at 130 g/L. Milk replacer was offered from day 4 of age twice a day (4 L each time) and was reduced to one meal on day 25 as starter intake was augmented. Form the second day of life, a commercial concentrate composed of sorghum grain, rolled corn, soybean meal, and cane molasses was offered from a bucket. The milk starter used was Premium Initiation Weaning Premature (Nuplen, Durango, México) with 13.0% moisture, 21.5% crude protein, 3.0% fat, 8.0% crude fiber, 7.0% ash, and 47.0% NFE. The starter concentrate was offered from the third day of age and was gradually increased.

Samples and Data Collection

Daily intake of milk and starter concentrate was recorded. The variation in consumption between days was evaluated as an indicator of stability. Feed conversion was estimated as the ratio of kg of dry matter (DM) intake/kg of BW gain, including DM from starter, milk replacer, and milk starter. Calves were evaluated daily following the morning milk feeding between 06:00 and 10:00 to check for diarrhea, pneumonia, otitis, and other diseases. On day 59 of the experiment, pre-prandial blood samples of all animals were collected from the jugular vein using vacutainer tubes with sodium citrate, EDTA, and without anticoagulant. These blood samples were maintained under refrigeration (4°C) until use. Tubes without anticoagulant were centrifuged (Sigma 2-16 k, Germany) at 3,500 x g for 20 min to obtain blood serum, which was stored in Eppendorf tubes and kept in a freezer at −20°C until analysis. Cholesterol, glucose, total protein, albumin, and bilirubin were determined with an autoanalyzer Kontrolab 2017. The blood sample collected in disodium-EDTA was used for complete blood count (CBC). The CBC leukocyte differential count and the hematocrit were determined in the hematology analyzer QS Kontrolab EasyVet.

Immunoglobulin Analyses

Calves were immunized against *Clostridium* spp. (Covexin 10 vaccine) on day 40 of the experiment, and serum obtained from the blood samples collected with sodium citrate was used to evaluate the antibodies against the antigen (*Clostridium* spp.). The antigen was bound to the microtiter plate and incubated overnight at room temperature. For antibody evaluation, 100 μL of the serum sample was added to each well and incubated (1 h, 37°C). Plates were decanted, and 100 μL of bovine IgG specific secondary antibody was added and incubated (1 h, 37°C).

Then, 100 μL of TMB (3.3′, 5.5′-tetramethylbenzidine liquid) was added to each well, and the reaction was stopped with 100 μL of H_2SO_4 (1%). The plates were analyzed in an ELISA reader (Model 350, BioRad) at a wavelength of 540 nm.

RNA Extraction From Blood Samples

Blood samples with sodium citrate from the control and the polyherbal treated group (4 g/d) were processed using SRL solution (Tris–HCl 10 mM pH 8, MgCl2 5 mM, and NaCl 10 mM) from a total volume of 24 mL of blood per experimental unit (calf). Total RNA was extracted from the leukocyte package using Trizol (Invitrogen). The RNA pellet was suspended in 20 μL of H_2O DEPC 0.1% water and stored at −80°C until processing. RNA purity was determined by electrophoresis on a 1% agarose gel. The RNA samples were treated with DNases (Promega). The purified RNA was quantified by spectrometry using A_{260}-A_{280} (21). Two pools of RNA (control and treatment) were prepared from eight biological repetitions (30 ug RNA) and stored at −70°C for 24 h and subsequently centrifuged for 15 min at 4°C.

Microarray Analyses

Microarray analyses were carried out at the Unidad de Microarreglos del Instituto de Fisiología Celular (UNAM) in Mexico City, using a heterologous mouse M22K_10_16 chip to evaluate the differential expression of the genes. The arrangement evaluated 24,341 genes, and 10 μg of total RNA as synthesized to cDNA with the incorporation of nucleotides dUTP-Alexa555 and dUTP-Alexa647, using the CyScribe First-Strand cDNA Amersham labeling kit. Fluorophore incorporation was analyzed at an absorbance of 555 nm for Cy3 and 655 nm for Cy5. Equal amounts of cDNA were hybridized using the HybIT2 solution (TeleChem International INC). The arrangements were incubated for 14 h at 42°C, with three consecutive washes using 1 x SCC, 0.05% SDS at room temperature. We used the equipment and software ScanArray 4000 (Packard BioChips) for acquisition and quantification of the images of the arrangements. Microarray data analysis was conducted with the genArise software (http://www.ifc.unam.mx/genarise/). The list of genes considered upregulated (Up) and downregulated (Down) was analyzed with the bioinformatics tool DAVID [Functional Annotation Bioinformatics Microarray Analysis, (https://david.ncifcrf.gov/)], which allows the grouping of genes based on their functional similarity.

Gene Ontology Analysis

Gene set enrichment analysis using gene ontology was applied to extract biological meaning from the identified differentially expressed transcripts; gene ontology terms with a *P*-value <0.05 were considered as enriched. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.8 Bioinformatic resource was used for gene set enrichment (22).

Validation of Differential Genes Expressed Using Quantitative Real-Time PCR

First-strand cDNA was synthetized from 1,500 ng of total RNA using Oligo(dT) and RevertAid Reverse Transcriptase (Thermo Scientific). Specific primers for PPARβ and GAPDH were designed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (**Table 1**). Primers for PPARα and ACOXI were taken from previous studies (**Table 1**) (23). The qPCR was performed on a real-time PCR system Rotor-GeneQ (Qiagen), using Sybr Green for detection (Thermo Scientific). Amplification for PPARα and ACOXI was carried out for one cycle at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 60°C for 60 s, and 70°C for 30 s. Amplification for PPARβ and Relb was carried out for one cycle at 95°C for 2 min, followed by 45 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control for normalization. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative mRNA quantification (24).

TABLE 1 | Sequences (5′ to 3′) of the primers used in the qPCR.

	Primers		
Gene	**Forward**	**Reverse**	**pb**
PPARα	CAATGGAGATGGTGGACACA	TTGTAGGAAGTCTGCCGAGAG	95
ACOX	TCCTACTGTGACCTCCATCAA	GGGTCCCAAGTTCACGAATAG	143
PPARβ	GTGGCTTCTGTTCACCGACA	GAAGTGAGTGCTCTGGTCCC	257
GAPDH	GCCATCACCATCTTCCAGG	GGTAGTGAAGACCCCAGTGG	96

Statistical Analyses

The Shapiro-Wilk test was used to test the normal distribution of variables. Data were analyzed using the R software (25), and orthogonal linear and quadratic polynomials were used to evaluate the effects of the polyherbal additive. The model used was: $Yij = \mu + \tau i + eij$, in which μ is the mean value, τi is the treatment effect (fixed), and *eij* is the error term. The initial body weight was used as a covariate in daily gain and final BW (26). The number of cases of diseases and the doses of antibiotics were analyzed using the Kruskal Wallis test. If the quadratic effect was significant, the optimal concentration for daily gain was estimated using the first derived from the quadratic equation (27).

RESULTS

Performance and Health Parameters in Calves

The animals treated with the polyherbal mixture showed a quadratic response in final BW, average daily gain (ADG), final hip height, and final thoracic girth, with the best numeric performance results with a dose of 4 g/d (**Table 2**). However, the optimal dose estimated by regression to maximum ADG was 2.47 ± 0.87 g/d of the polyherbal mixture. Starter intake was stimulated at a dose of 4 g (quadratic $P < 0.001$), whereas liquid ingestion showed a linear increase ($P < 0.001$), compensating for the reduction in solid consumption with no effect on feed conversion (**Table 2**). All calves received colostrum following birth, and immunoglobulins in the blood were measured by refractometry, the results obtained showed a concentration of

TABLE 2 | Effects of the polyherbal mixture treatment on growth performance and health status.

	Polyherbal mixture (g/d)				SEM	*P*-value	
	0	3	4	5		Linear	Quadratic
Average daily gain g/d	0.672	0.763	0.772	0.653	0.0206	0.89	0.008
Final BW kg	86.74	92.64	93.28	85.50	1.3904	0.78	0.008
Final wither height cm	90.06	91.18	91.33	90.51	0.5281	0.72	0.30
Final hip height cm	95.42	97.11	97.34	95.39	0.4268	0.53	0.05
Final thoracic girth cm	101.56	105.01	105.35	103.57	0.6006	0.20	0.02
Starter intake g/d	829	948	889	677	31.4816	0.02	0.001
Feed intake variation among individuals %	107.38	108.13	112.66	113.71	1.4026	0.12	0.96
Milk + replacer intake liters/d	2.454	2.564	2.582	2.666	0.0188	0.0001	0.63
Feed conversion ratio	2.15	2.06	1.94	2.07	0.0501	0.32	0.19
No. diarrheas	0.29	0.24	0.40	0.01	0.0595	0.81	0.70
No. pneumonias	3.61	0.50	3.20	0.88	0.5535	0.34	0.80
No. otitis	5.66	1.20	2.40	4.77	1.0023	0.79	0.06
Antibiotic doses	5.35	1.54	4.32	2.33	0.9383	0.41	0.60
Immunoglobulins (Anti-Clostridium IgG)	1.496	1.438	1.851	1.764	0.0766	0.07	0.62

SEM, standard error of the mean.

TABLE 3 | Effects of the polyherbal mixture on blood chemistry parameters.

	Polyherbal mixture (g/d)				SEM	*P*-value	
	0	3	4	5		Linear	Quadratic
Glucose mmol/L	4.101	2.975	3.480	2.621	2.5144	0.0001	0.47
B-OH Butyrate mmol	0.329	0.250	0.300	0.355	0.0156	0.35	0.03
Urea mmol/L	7.87	6.07	6.42	6.42	0.7066	0.08	0.09
Uric acid mmol/L	30.09	36.87	33.90	36.34	0.0276	0.24	0.49
Creatinine μmol/L	89.46	91.05	93.70	94.23	0.0309	0.60	0.94
Total protein mmol/L	69.9	69.2	70.8	70	0.1547	0.14	0.70
Globulin g/dL	3.04	2.76	2.76	2.90	0.0853	0.44	0.09
Albumin g/dL	3.95	4.16	4.32	3.99	0.1030	0.59	0.02
Ratio A/G	1.33	1.53	1.60	1.37	0.0563	0.59	0.01
Cholesterol mmol/L	24.26	22.91	22.85	23.72	2.7943	0.73	0.33
Bilirubin mmol/L	0.370	0.470	0.490	0.511	0.0211	0.01	0.34
ALP U/L	25.71	28.60	25.60	26.11	0.9695	0.72	0.98
LDH U/L	79.65	68.20	75.20	78.89	2.6029	0.85	0.21
AST (GOT) U/L	25.53	20.10	21.90	21.22	1.2873	0.45	0.49
Calcium mmol/L	2.30	2.22	2.52	2.29	0.2478	0.43	0.36
Phosphorus mmol/L	1.66	1.52	1.62	1.53	0.1352	0.32	0.69

ALP, alkaline phosphatase; LDH, lactate dehydrogenase; AST (GOT), aspartate aminotransferase; SEM, standard error of the mean.

6.54± 0.525 g/dl. A reduction in otitis (quadratic effect $P < 0.10$) was observed with intermediate doses of polyherbal in the calves (**Table 2**), and a linear increment ($P < 0.10$) of anti-*Clostrdium* spp. IgG was detected by enzyme-linked immunosorbent assay.

Blood Chemistry and Biometry

Blood chemistry changes are shown in **Table 3**. The treatment with polyherbal mixture caused a reduction in glucose levels (linear $P < 0.01$), with a quadratic increase in B-OH butyrate ($P < 0.05$). Globulin levels were reduced ($P < 0.10$ quadratic) and albumin levels showed a quadratic increment ($P < 0.05$), resulting in a reduction in the albumin globulin ratio (quadratic $P < 0.01$). Urea showed a reduction ($P < 0.10$), whereas uric acid was not affected. The bilirubin level increased linearly ($P < 0.01$) with the polyherbal treatment; however, liver enzymes [alkaline phosphatase, ALP; lactate dehydrogenase, LDH; and aspartate aminotransferase, AST (GOT)] were not affected. Serum calcium and phosphorus remained unchanged. The results of the biometry are presented in **Table 4**. There was a reduction in neutrophils in band ($P < 0.05$).

TABLE 4 | Effects of the polyherbal mixture on blood parameters.

	Polyherbal mixture (g/d)				SEM	*P*-value	
	0	3	4	5		Linear	Quadratic
Hematocrit %	35.13	34.7	35.8	36.05	0.335	0.37	0.72
Hemoglobin g/dl	11.91	11.7	12.15	12.08	0.116	0.48	0.82
Erythrocytes $\times 10^6$/ml	5.39	5.16	5.35	5.39	0.072	0.77	0.38
Mean corpuscular volume fl	65.73	67.29	66.82	66.86	0.576	0.58	0.54
Mean corpuscular Hemoglobin pg	22.27	22.71	22.68	22.4	0.209	0.85	0.41
Mean corpuscular hemoglobin concentration g/dl	33.9	33.72	33.94	33.51	0.111	0.25	0.53
Platelets $\times 10^3$/ml	407.22	376.20	449.1	458.22	13.912	0.09	0.52
Wintrobe sedimentation rate ml/h	0.27	0	0	0.55	0.150	0.58	0.24
Leucocytes $\times 10^3$/ml	11.4	11.57	12.4	11.05	0.391	0.96	0.49
Lymphocytes $\times 10^3$/ml	45.88	28.80	44.70	40.00	2.134	0.91	0.08
Monocytes $\times 10^3$/ml	4.55	3.90	5.00	4.66	0.253	0.56	0.77
Neutrophils segmented $\times 10^3$/ml	41.94	62.9	46.40	49.00	2.328	0.77	0.01
Neutrophils in band $\times 10^3$/ml	4.33	1.70	1.60	2.44	0.381	0.02	0.003
Eosinophils $\times 10^3$/ml	3.0	2.6	2.3	3.66	0.280	0.53	0.16
Basophiles $\times 10^3$/ml	0.27	0.10	0.00	0.22	0.055	0.59	0.09
Plasma proteins g/dL	9.09	8.64	8.98	9.42	0.115	0.22	0.07

SEM, standard error of the mean.

Lymphocytes, basophiles, and plasma protein were reduced with lower or intermediate doses of BioCholine (quadratic effect, $P < 0.10$).

Gene Expression Variations

The heterologous microarray represented ∼22,000 transcripts, of which 2,442 were differentially expressed; a total of 1,093 and 1,349 transcripts were up- and downregulated, respectively. A total of 264 genes with values of +2.0 to 5.5 were upregulated and 401 genes with values of −2.0 to −5.8 were downregulated. The 30 most strongly up- and downregulated transcripts in blood leukocytes of weaning calves supplemented with polyherbal mixture are presented in **Tables 5**, **6**. The most strongly upregulated transcripts include functional categories such as proto-oncogene (*Jun*, *Lck*, *Pdgfra*), tyrosine protein kinase (*Tyro3*, *Lck*, *Pdgfra*), immunoglobulin domain (*Tyro3*, *Lrn2*, *Pdgfra*, *Pdgfrl*), and ATP-binding (*Abcc8*, *Tyro3*, *Entpd2*, *Lck*, *Pdgfra*, *Plk1*) (**Table 5**). The most strongly downregulated transcripts include functional categories such as phosphoprotein (*Erc1*, *Rab11fip2*, *Rpusd2*, *Sh3d19*, *Tal1*, *Baz1a*, *Cobl*, *Cry2*, *Dapk1*, *Glycam1*, *Grhpr*, *Hmg20b*, *Kif2c*, *Lig1*, *Polr3d*, *Ptpn6*, *Sel1*, *Xirp2*), protein transport (*Erc1*, *Rab11fip2*, *Xpo5*, *Serp1*), and ubiquitin-like modifier conjugation (*Tal1*, *Cry2*, *Dapk1*, *Hmg20b*, *Kif2c*, *Polr3d*) (**Table 6**).

Gene Set Enrichment Analysis (GSEA) of upregulated genes (**Table 7**) revealed that the three biological processes with higher fold change were tight junction (Fc: 3.7; $P = 0.00002$), mucin type O-Glycan biosynthesis (Fc: 3.7; $P = 0.044$), and intestinal immune network for IgA production (Fc: 3.4; $P = 0.015$). We observed reduced expression of genes related with tight junction (*Rab13*, *Src*, *Afdn*, *Cdc42*, *Llgl2*, *Mpp5*, *Myh10*, *Myh9*, *Myl9*, *Pard6g*, *Prkci Tjp1*); among these genes, there were four code for claudins (*Cldn-7*, *Cldn8*, *Cldn9*, *Cldn16*). The expression of *gcnt3* gene was reduced (Fc = −1.6); this gene is coded to the enzyme responsible for synthesis of all known β6 N-acetylglucosaminides. The treatment with polyherbal mixture reduced the expression of cytokines necessary to promote terminal differentiation and switching of immunoglobulin A class of B cells, explaining the downregulation of the genes involved in the intestinal immune network for IgA production (Cd40lg, Cd86, H2-DMb1, Il10, Madcam1, Tgfβ1, Tnfrsf13b). The microarray did not identify differentially expressed transcripts that codified for *Tnf-α*, *Ifn*, *Il-6*, and *Il8*, which code for inflammatory cytokines. In contrast, a reduced expression of *Tgfβ1* (Fc: −3.4) and *Il-100* (Fc: −2.5) was shown.

Analysis of gene enrichment with positive regulation identified pantothenate and CoA biosynthesis ($P = 0.03$), arachidonic acid metabolism ($P = 0.01$), and diabetes mellitus type 2 ($P = 0.01$) (**Table 7**). The treatment with polyherbal mixture increased the expression of four genes whose transcription is related to pantothenate and CoA biosynthesis (*Enpp1*, *Enpp3*, *Pank4*, and *Vnn3*).

Analysis of total up and downregulated transcripts (**Figure 1**), indicates an important modulator effect of polyherbal mixture treatment on *Pelp1* modulation of estrogen receptor activity (Fold change, Fc = 4.4; *Src*, *Esr1*, and *Mapk1*), nuclear receptors in lipid metabolism and toxicity (Fc = 2.4; *Abca1*, *Abcb1b*, *Cyp1a2*, *Cyp27b1*, *Nr1h3*, *Nr1i3*, *PPARa*, *PPARd*, *Rara*, and *Rarg*), and tight junction (Fc: 2.4).

TABLE 5 | Microarray analysis of differential gene expression profile in peripheral blood cells (upregulated genes).

Gene symbol	Gene description	Fold change[a]
Plk1	Polo-like kinase 1	5.5
Col11a1	Collagen, type XI, alpha 1	4.9
Tyro3	Protein tyrosine kinase 3	4.9
Klhdc9	Kelch domain containing 9	4.8
Casp2	Caspase 2	4.5
Entpd2	Ectonucleoside triphosphate diphosphohydrolase 2	4.2
Mcur1	Mitochondrial calcium uniporter regulator 1	4.1
Acoxl	Acyl-Coenzyme A oxidase-like	4.0
Lrrn2	Leucine rich repeat protein 2, neuronal	4.0
Snrpc	U1 small nuclear ribonucleoprotein C	3.9
Klra20	Killer cell lectin-like receptor subfamily A, member 20	3.9
Pdgfra	Platelet derived growth factor receptor, alpha polypeptide	3.9
Etd	Embryonic testis differentiation	3.8
Zfp27	Zinc finger protein 27	3.6
Prdx6	Peroxiredoxin 6	3.6
Abcc8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	3.6
Elovl2	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2	3.6
Jun	Jun proto-oncogene	3.5
Rab3gap1	RAB3 GTPase activating protein subunit 1	3.5
Ccser2	Coiled-coil serine rich 2	3.5
Dsc1	Desmocollin 1	3.5
Mrps5	Mitochondrial ribosomal protein S5	3.4
Mga	MAX gene associated	3.4
TGF-β	transforming growth factor, beta 1	3.4
Defa14	Defensin, alpha, 14	3.3
Cenpp	Centromere protein P	3.3
Rpl41	Ribosomal protein L41	3.3
Pdgfrl	Platelet-derived growth factor receptor-like	3.3
Lck	Lymphocyte protein tyrosine kinase	3.2
Vpreb3	Pre-B lymphocyte gene 3	3.2

[a] *Polyherbal mixture treated vs. no treated animals.*

TABLE 6 | Microarray analysis of differential gene expression profile in peripheral blood cells (downregulated genes).

Gene symbol	Gene description	Fold change[a]
Hmg20b	High mobility group 20B	−5.8
Cyp21a1	Cytochrome P450, family 21, subfamily a, polypeptide 1	−4.5
Ptgdr	Prostaglandin D receptor	−4.3
Serp1	Stress-associated endoplasmic reticulum protein 1	−4.3
Baz1a	Bromodomain adjacent to zinc finger domain 1A	−4.2
Cry2	Cryptochrome 2 (photolyase-like)	−4.2
Psmd8	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	−4.2
Slc22a15	Solute carrier family 22 (organic anion/cation transporter), member 15	−4.2
Grhpr	Glyoxylate reductase/hydroxypyruvate reductase	−4.1
Kif2c	Kinesin family member 2C	−4.1
Rpusd2	RNA pseudouridylate synthase domain containing 2	−4.1
Cobl	Cordon-bleu WH2 repeat	−4.1
Glycam1	Glycosylation dependent cell adhesion molecule 1	−4.1
Xpo5	Exportin 5	−4.0
Dapk1	Death associated protein kinase 1	−4.0
Sel1l	Sel-1 suppressor of lin-12-like	−4.0
Xirp2	Xin actin-binding repeat containing 2	−4.0
Sh3d19	SH3 domain protein D19	−3.9
Lzic	Leucine zipper and CTNNBIP1 domain containing	−3.9
Rab11fip2	RAB11 family interacting protein 2 (class I)	−3.9
Erc1	ELKS/RAB6-interacting/CAST family member 1	−3.8
Lig1	Ligase I, DNA, ATP-dependent	−3.8
Wif1	Wnt inhibitory factor 1	−3.8
Tal1	T cell acute lymphocytic leukemia 1	−3.8
Gsc2	Goosecoid homebox 2	−3.8
Mc5r	Melanocortin 5 receptor	−3.8
Ptpn6	Protein tyrosine phosphatase, non-receptor type 6	−3.8
Polr3d	Polymerase (RNA) III (DNA directed) polypeptide D	−3.8
Grpr	Gastrin releasing peptide receptor	−3.8
G6pc3	Glucose 6 phosphatase, catalytic, 3	−3.8

[a] *Polyherbal mixture treated vs. no treated animals.*

Analysis of gene expression by qPCR (**Table 8**) indicates that PPARα and PPARβ gene transcription were upregulated ($P = 0.15$) and downregulated ($P = 0.05$), respectively.

DISCUSSION

Calf Parameters

The polyherbal mixture provides conjugates of choline (mainly PCho) and secondary metabolites that, at low doses, have nutraceutical properties (11), which explains the improved response in growth (quadratic effect). Growing calves have a high protein synthesis, and choline participates as a methyl donor (via betaine) necessary for the methylation of DNA, RNA, and proteins (28, 29), in addition to the synthesis of the phospholipids of cell membranes and plasma lipoproteins (30) and to multiple functions in the organism (31). Few choline evaluations have been reported in calves since deficiencies of this nutrient were reported in the 1950s (29). Although it is included in milk replacers and starters, as suggested by the NRC (32), it is not supplemented after weaning. However, studies with growing lambs show a quadratic response to supplementation in the form of rumen-protected choline (RPC) or with the polyherbal mixture evaluated here (11, 12, 30). The positive response to choline is due to the rapid growth rate of Holstein cattle after weaning, and genetic selection can lead to increased requirements for this nutrient. Some RPC assessments have been made in steers in growing and finishing stages. In these studies, it was shown that when the animals approach to mature weights, the response to the nutrient is lower. Response to RPC was also reduced as the animal approaches mature weight (33–35).

In calves, few phytogenic feed additives with essential oils have been evaluated (36), which report higher intake of starter and better feed efficiencies and gain, whereas other authors did not find intake responses (37, 38). It is not clear if the changes in consumption showed in this investigation were due to some secondary metabolites of polyherbal mixture or by the conjugates of choline. In lambs that responded to the same polyherbal mixture in ADG, intake was not affected (12). However, in an experiment with graded levels of RPC, intake showed a quadratic response parallel to the gain response (39). Changes in thoracic girth in intermediate doses indicate greater rumen development, but there are few reports where nutrients in the diet affected these variables, and generally, those changes are not noticeable (40–42).

Regarding the evaluated health parameters, the reduction in otitis can be explained by some antibacterial metabolites in the polyherbal mixture (11), by the choline antioxidant capacity (43) that may play a preventive role in otitis (44), or by improving the immune response (45); this is important as in calves, otitis is commonly linked to pneumonia (46). However, more studies are necessary to confirm these results. The polyherbal mixture improved the immune response to vaccination, as observed in the quantifications of serum immunoglobulins in some feed plant additives (8), and could be related with the upregulation of some genes such as *Tyro3*, *Lrn2*, *Pdgfra*, and *Pdgfrl*.

TABLE 7 | Gene ontology enrichment analysis in peripheral blood cells.

GO biological process term	Count[a]	*P*-Value	Fold change[b]
Pantothenate and CoA biosynthesis	4	0.03	5.7
Tight junction	16	0.00002	3.7
Type II diabetes mellitus	7	0.031	3.6
Intestinal immune network for IgA production	7	0.015	3.4
Arachidonic acid metabolism	10	0.01	2.9
Neuroactive ligand-receptor interaction	23	0.019	1.7
Adherents junction	9	0.022	2.6
Ribosome	14	0.023	2.0
ECM-receptor interaction	10	0.025	2.3
Pathways in cancer	28	0.041	1.5
Focal adhesion	17	0.042	1.7
Mucin type O-Glycan biosynthesis	5	0.044	3.7
Endocytosis	20	0.047	1.6

[a]*Number of genes associated.*
[b]*Polyherbal mixture treated vs. no treated animals.*

Blood Chemistry and Biometry

The blood chemistry values were in the normal range for calves (47, 48). Only glucose showed a slight reduction in animals supplemented with herbal choline. The polyherbal mixture contains *Azadirachta indica*, which has shown hypoglycemic effects (49). However, these effects do not affect leukocytes, which was indicated by the downregulation of the expression (−3.8 fold lower than in the control group) of the *g6pc3* gene, which codes for glucose-6-phosphatase involved in the homeostatic regulation of glucose in monocytes and neutrophils. The major function of glucose 6-phosphatase-β is to provide recycled glucose to the cytoplasm of neutrophils to maintain normal function (50).

The change in butyrate may be an indicator of ruminal development. Glucose is reduced because as calves are growing,

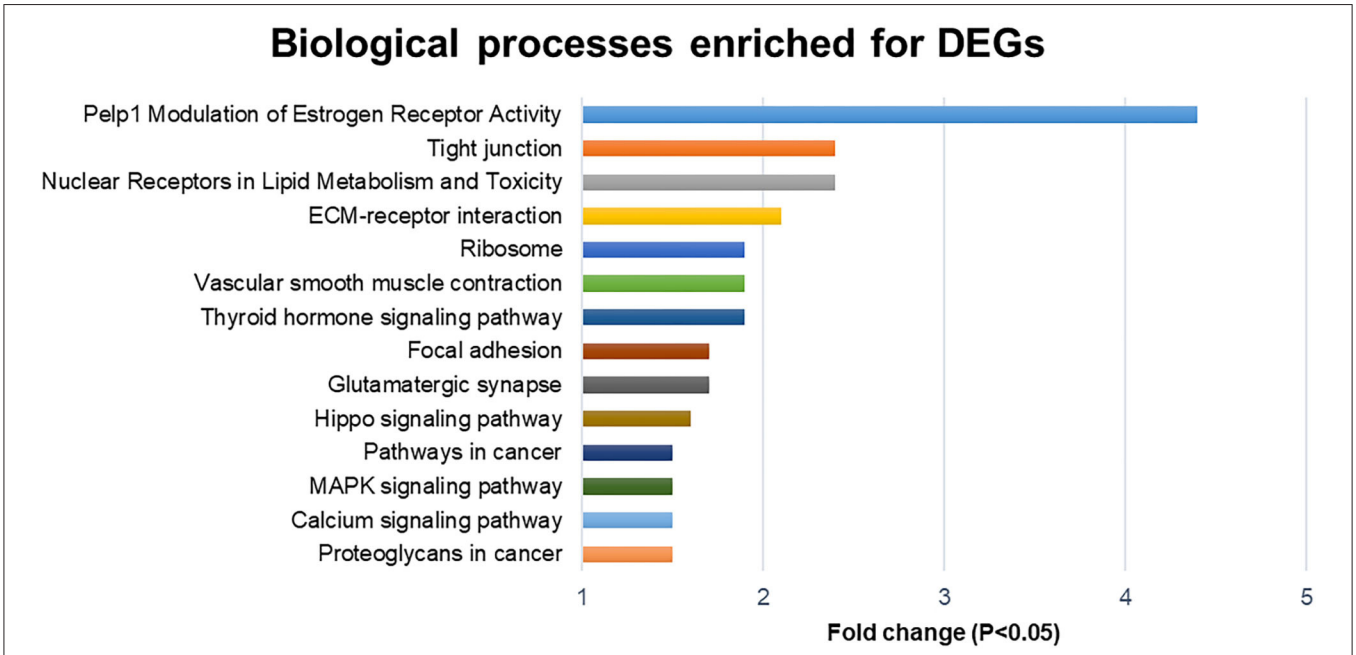

FIGURE 1 | Biological processes enriched for differentially expressed genes (DEGs; down and upregulated transcripts).

TABLE 8 | Analysis of gene expression ($^{\Delta\Delta}$CT).

	Polyherbal mixture (g/d)						
Gene	**0**	**3**	**4**	**5**	**SEM**	**Linear**	**Quadratic**
PPARα	1.24	21.57	14.84	42.49	6.35	0.53	0.15
PPARβ	3.33	2.99	0.82	0.81	0.59	0.84	0.05

PPARα, peroxisome proliferator activated receptor α; PPARβ, peroxisome proliferator activated receptor β/δ; SEM, standard error of mean.

they depend more on volatile fatty acids. Serum calcium and phosphorus also indicate that the feed additive did not affect the mineral balance of these elements, regardless of differences in feed intake of starter (51, 52). The calves were healthy, and minor changes in some metabolites were detected, which were, however, not associated with infectious agents or chronic inflammatory processes. Urea blood reductions indicate an adequate general situation of protein metabolism (53), confirmed by uric acid, a metabolic product of protein metabolism; the creatinine levels indicate that the kidney function was not affected (54).

The main serum indicators used to evaluate liver alterations include AST(GOT), ALT, bilirubin, total protein, and total albumin (55) and were not altered by the treatment with polyherbal mixture, confirming that the liver was not damaged. Changes in bilirubin levels can be explained by the reduction in the expression of *ugt2b37* (UDP-glucuronosyltransferase), which codes for the enzyme involved in bilirubin metabolism (56) and catalyzes the transformation of unconjugated or indirect bilirubin into conjugated or direct bilirubin by the addition of glucuronic acid. Some herbal supplements and extracts with polyphenolic compounds can have inhibitory effects on the gene responsible for glucoronidation of bilirubin (57). Since the calves in our study were healthy, presumably cross-reactions from some metabolites from herbal choline could be responsible for the increase in bilirubin, rather than liver injury. According to a previous study, serum bilirubin concentration is not considered as a sensitive indicator of liver disease in young calves (55).

In other experiments with feed plant additives, serum protein levels were not affected (36), and the results may vary depending on the mechanism of action of phytobiotics, secondary metabolites, and doses. Liver enzymes as ALT is the most specific test to detect liver dysfunction as well as LDH (55), suggesting that the polyherbal mixture evaluated does not have hepatotoxic metabolites and its choline conjugates could have an antioxidant effect (58) and other beneficial effects for liver function of this nutrient (59). The Polyherbal mixture has several chemical compounds, and some of them have already been reported to possess bactericidal properties (11, 12). Therefore, this could affect the structure and composition of the microbiota, affecting the ruminal fermentation. These could explain the lack of correlation between the starter intake and blood B-OH butyrate.

The biometry results indicate that calves were healthy and within the reported values (60, 61), similar to those shown for 1-month-old calves (62). The reduction in neutrophils in band was not correlated with the presence of bacterial disease (63). In fact, the polyherbal mixture treatment stimulated genes such as *Defa14*, recognized as antimicrobial peptides (64). In one experiment, aflatoxin B1 decreased neutrophils in calves, and the administration of choline helped to reduce the toxic adverse effects (65).

Gene Expression Variations

Microarray analysis allowed us to detect significant changes in gene expression induced by metabolites in the polyherbal mixture. The analysis of changes in gene expression by bioinformatics analysis (GSEA) resulted in the identification of specific metabolic processes involved in calf metabolism. Although this work only evaluates gene expression in leukocytes, the variations in the expression observed in these cells indicates variations produced in other cells.

The changes in gene expression could explain some variations observed in calf performance and changes in blood variables. The possible physiological implication of this variation raises important questions about the plant metabolites. For example, the reduction in the expression of *gcnt3* gene and its implications may be important since this gene coded the enzyme responsible for the synthesis of all β6 N-acetylglucosaminides, which play an important role in O-linked glycosylation in mucin biosynthesis and, consequently, has implications on the functional state of the mucosa of the intestinal and respiratory tracts (66).

In calves treated with polyherbal mixture, the expression of Claudin genes in leukocytes was reduced. Claudins are the main transmembrane proteins that regulate the intestinal epithelial permeability, controlling ion and microbial product diffusion through the epithelial cell layer (67, 68). Malmuthuge et al. (69) have reported that the diet regulates the expression of Claudin 4 and occludin, altering the absorption of macromolecules. Considering that modification in expression of these genes was induced by metabolites in the polyherbal mixture, the same response could be present in cells of the gastrointestinal tract, thereby modifying intestinal permeability and the absorption of macromolecules.

The genetic enrichment analysis provided a perspective on the changes induced by the polyherbal treatment at different metabolic stages. Increased pantothenate and CoA biosynthesis (**Table 7**) has important consequences on energy metabolism, which were manifested in calf growth, and expression of *Pank4*, which encodes pantothenate kinase, a regulatory enzyme in CoA biosynthesis. The gene *Pank4* phosphorylates the vitamin pantothenate to 4′-phosphopantothenate, which is converted into CoA and participates in the tricarboxylic acid cycle, fatty acid metabolism, and numerous other reactions of intermediary metabolism (70). The *Pank4* mRNA is upregulated

at high glucose concentrations to allow higher flux through the tricarboxylic acid cycle (71).

Coenzyme A biosynthesis and the increased PPARα gene expression induced by choline have important implications. Here, PPARα stands out because the group of nuclear proteins called PPAR (peroxisome proliferator-activated receptor) plays a key role in the regulation of metabolic pathways such as lipid metabolism (72), and it is possible that the over-expression of the genes *Acoxl* (acetyl CoA-oxidase) and the gene *Cpt2* (carnitine palmitoyl transferase 2) is mediated by the positive regulation of PPARα and by other effects of choline, such as antioxidant effects, lipotropic effects, or through the activity of *GSH-Px* (43).

The treatment with polyherbal mixture also stimulated a group of genes associated with nuclear receptors in lipid metabolism and toxicity (**Figure 1**). There are PPAR receptors for fatty acids and *cytochrome P450* genes involved in lipid metabolism; some genes of *cytochrome P450* are related to the detoxification of environmental toxins and the metabolism of xenobiotics and drugs (73); this explains the beneficial effects of choline in calves with aflatoxins (65). The ECM-receptor interaction pathway is involved in adipose tissues and important for adipogenesis and functional macromolecules (glycosaminoglycans, collagen, elastin, fibronection, and lammin), but there are complex interactions with other cells, which need to be addressed (74).

Ribosomal gene stimulation suggests that polyherbal treatment simulates protein synthesis, which coincides with the growth response observed in calves; the ribosomes are the cellular organelles responsible for protein synthesis in cells (75). The effects of glutamatergic synapse pathway stimulation in calves are unclear, but it is known that glutamate is an important neurotransmitter in the central nervous system and that its stimulation affects receptors, channels, and neurotransmitter transporters (76), with potential effects on the stress response. In their review, Musazzi et al. (77) present evidence that glucocorticoids and acute stress increase extracellular glutamate levels, affecting glutamate release in the brain; the authors highlight that chronic stress may reduce glutamate metabolism. In this sense, animals in confinement can be chronically stressed, and calves receiving a dose of 3 g/d showed better health and improved weight gain. This leads us to infer that this route could have reduced stress.

Focal adhesion processes may have improved the health conditions of calves since they participated in cell migration and invasion (78). Liu et al. (79) have demonstrated that the phytoestrogen genistein at high concentrations reduced the damage in estradiol-induced cardiovascular cells, affecting the focal adhesion pathway. The Hippo pathway plays a role in organ size and morphogenesis control as well as cancer development (80). Growth is an important parameter in calves, and changes in morphometric measurements were observed in this study.

The overall effects reflect the combined effects of conjugates of choline and secondary metabolites from polyherbal mixture (11) on the metabolism of lipids and glucose (59, 81) and on the immune response (13, 17, 18); this is in agreement with the demonstrated beneficial effects of natural products from traditionally used medicinal plants (72), and it can have been relative compared with the gene expression in experiments with synthetic choline products (82).

The treatment with polyherbal mixture could have an important effect in PPARα gene expression. It is important for immunity in calves because it is predominantly expressed in T and B cells (83). Also, PPARα is an important regulator of gene expression in rumen epithelium during the period from pre-ruminant to ruminant and can be involved in the mediation of energy metabolism within the rumen epithelium to support rumen development and differentiation of the ruminal papillae during weaning (84, 85). Metabolically, PPAR receptors induce the proliferation of peroxisomes in cells, a process that generates the transcription of the acyl-CoA oxidase (*AcoxI*) gene (86); however, *AcoxI* gene validation by qPCR did not confirm the microarray results where *AcoxI* was upregulated. On the other hand, the increased PPARα gene transcription (**Table 8**) can be explained by the mechanism of action of choline conjugates, mainly phosphatidylcholine, which we assume is the main metabolite involved in the stimulation of gene expression of *Pla2g6*, *Pla2g4e*, and *Pla2g2d*, which encode phospholipases A2, involved in the hydrolysis of arachidonic acid in the C2 position of phosphatidylcholine (87). The release of arachidonic acid stimulates the expression of the *Ptgds* and *Ptgis* genes, which encode enzymes involved in the cyclooxygenase pathway for the production of prostaglandins and prostacyclines. The genes *Alox12* and *Alox15* encode enzymes involved in the lipoxygenase pathway, synthesizing the acids 12-HPETE (12-hydroperoxyeicosatetraenoic acid), 15-HPETE (15-hydroperoxyeicosatetraenoic acid), and Cyp4a10, which encode monooxygenase to synthesize 20-hydroxyeicosatetraenoic acid (20-HETE). These fatty acids derived from arachidonic acid are incorporated into the β-oxidation process, but they also act as agonists of the expression of the PPARα gene (88), a transcription factor that reduces the inflammatory response by sub-regulation of genes induced by cytokines, an effect attributed to the direct interaction of PPARα with the p65 subunit of *NF-kB*, reducing its link with DNA (89). This would explain the reduction in the expression of *Rel* and *RelA* genes of *NF-kB*. The absence of differentially expressed genes (*Tnfα*, *Ifn*, *Il2*, *Il6*, *Il8*) that code for proinflammatory cytokines and the reduced production of profibrotic factors such as *Tgf-β1* (**Table 5**) would also explain the reduction in the expression of anti-inflammatory interleukin 10 gene in calves (90).

In addition, the activation of the transcription factor PPARα by arachidonic acid or other metabolites derived from arachidonic acid, such as 20-HETE, and the binding with its receptor cis-9 retinoic acid reduced the expression of the *Scd1*, *Nr1h3*, *Gyk2*, and *Aqp7* genes, involved in gluconeogenesis, lipogenesis, and cholesterol metabolism, and increased the expression of genes *Acsl4*, *Cyp4a1*, *Acoxl*, *Cpt2*, and *Mmp10*, involved in fatty acid transport and oxidation and in adipocyte differentiation (90, 91).

In contrast to PPARα, the downregulation of PPARβ gene transcription is explained for a specific agonist. All PPAR receptors have a ligand-binding domain (LBD), enabling PPAR activation by polar structure ligands, particularly by fatty acids (91). Arachidonic acid (AA) and AA metabolites connect with LBD and act as natural activators of PPARα (92). However,

Naruhn et al. (93) identified that 15-HETE strongly induces the expression of PPARβ and, conversely, inhibition of 15-HETE synthesis reduces PPARβ transcriptional activity. In this sense, upregulated genes encode for lipoxygenases that synthetize 12-HPETE and 15-HPETE (arachidonate 12-lipoxygenase and arachidonate 15-lipoxygenase), but microarray did not identify differentially expressed genes that encoded for glutathione peroxidase (Gpx), responsible for the synthesis of 12-HETE and 15-HETE from 12-HPETE and 15-HPETE.

In weaning calves, stress triggers inflammatory responses (94). Prolonged stress suppresses immune functions to enable survival, consequently increasing disease susceptibility (95). Lewis et al. (45) have shown that phosphatidylcholine can stimulate the immune response, resulting in up-regulated PPARα gene activation, modulating the pro-inflammatory response and preventing its excessive activation (91). Optimizing calf health from birth to weaning will impact long-term health and animal welfare (96), allowing the animals to perform better in their first calving and lactation (97).

CONCLUSIONS

The polyherbal mixture treatment, at doses of 4 g/d, could improve growth and health status during the pre-ruminant to the weaning period through modification of gene expression. Gene expression analysis confirmed that polyherbal treatment could improve the metabolism of lipids, carbohydrates, proteins, and also immune response. However, more studies are necessary to provide evidence of this aspect. Our results confirm the usefulness of plant compounds in animal feed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Academic Committee of Doctorado en Ciencias Agropecuarias at Universidad Autónoma Metropolitana- Xochimilco, Mexico City, Mexico.

AUTHOR CONTRIBUTIONS

CD and AG performed the experiments and wrote section of the manuscript. EM and DM contributed to conception of the study, participated in its design, and helped to draft the manuscript. GM and PH participated in the design of the experiments and performed the statistical analysis. MP carried out the immunoassays. EE and AL participated in the coordination of the fieldwork. LV carried out the animal care. All authors contributed to manuscript revision, read, and approved the submitted version.

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Impact of Zinc and/or Herbal Mixture on Ruminal Fermentation, Microbiota, and Histopathology in Lambs

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We investigated the effect of diets containing organic zinc and a mixture of medicinal herbs on ruminal microbial fermentation and histopathology in lambs. Twenty-eight lambs were divided into four groups: unsupplemented animals (Control), animals supplemented with organic zinc (Zn, 70 mg Zn/kg diet), animals supplemented with a mixture of dry medicinal herbs (Herbs, 100 g dry matter (DM)/d) and animals supplemented with both zinc and herbs (Zn+Herbs). Each lamb was fed a basal diet composed of meadow hay (700 g DM/d) and barley (300 g DM/d). The herbs *Fumaria officinalis* L. (FO), *Malva sylvestris* L. (MS), *Artemisia absinthium* L. (AA) and *Matricaria chamomilla* L. (MC) were mixed in equal proportions. The lambs were slaughtered after 70 d. The ruminal contents were used to determine the parameters of fermentation *in vitro* and *in vivo* and to quantify the microbes by molecular and microscopic methods. Samples of fresh ruminal tissue were used for histopathological evaluation. Quantitative analyses of the bioactive compounds in FO, MS, AA, and MC identified 3.961, 0.654, 6.482, and 12.084 g/kg DM phenolic acids and 12.211, 6.479, 0.349, and 2.442 g/kg DM flavonoids, respectively. The alkaloid content in FO was 6.015 g/kg DM. The diets affected the levels of total gas, methane and *n*-butyrate *in vitro* ($P < 0.046$, < 0.001, and < 0.001, respectively). Relative quantification by real-time PCR indicated a lower total ruminal bacterial population in the lambs in the Zn and Zn+Herbs groups than the Control group ($P < 0.05$). The relative abundances of *Ruminococcus albus*, *R. flavefaciens*, *Streptococcus bovis,* and *Butyrivibrio proteoclasticus* shifted in the Zn group. Morphological observation found a focally mixed infiltration of inflammatory cells in the lamina propria of the rumen in the Zn+Herbs group. The effect of the organic zinc and the herbal mixture on the parameters of ruminal fermentation *in vitro* was not confirmed *in vivo*, perhaps because the ruminal microbiota of the lambs adapted to the zinc-supplemented diets. Long-term supplementation of a diet combining zinc and medicinal herbs, however, may negatively affect the health of the ruminal epithelium of lambs.

Keywords: bacteria, ciliated protozoa, hematological profiles, histology, phytochemicals, sheep

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INTRODUCTION

The ruminal microbial fermentation of dietary substrates plays a main role in the ability of ruminants to use fibrous dietary substrates but is also associated with emissions of methane and the excessive excretion of nitrogen in manure. Modern animal production systems require the maintenance of optimal animal health and the safe and efficient production of high-quality animal products. Understanding the ruminal microbiome in all aspects of bacterial, archaeal or eukaryotic populations and in all factors for manipulating the microbiome to maximize productivity while decreasing negative environmental impacts is therefore necessary (1, 2).

Our previous results indicated that replacing 10% of meadow hay with different mixtures of dry medicinal plants could influence the patterns of fermentation (3, 4). Recent findings with plant nutraceuticals highlight the dependence of the effect of dry medicinal plants on the variety and synergy of plant polyphenols and the combination of bioactive compounds, which together affect and contribute to a specific pharmacological efficacy (5, 6). Zinc plays catalytic, structural and regulatory roles for enzymes, proteins and transcription factors and is thus a key trace element for improving immunological functions (7, 8). The bioavailability of zinc in the diets of ruminants also depends on the chemical form, content and interaction of zinc with dietary constituents (9, 10). The most recent requirements and recommendations for dietary zinc in ruminants vary between 40 and 130 mg/kg DM of the complete diet (11). The organic forms of trace elements bound by organic ligands should be more resistant to interactions in the ruminant digestive tract and can be more bioavailable than inorganic sources (12). The rumen of ruminants allows the selective uptake of nutrients generated by intraruminal microbial fermentation because the rumen is covered by a stratified epithelium that consists of leaflike papillae, which greatly increase the area and size of the absorptive surface (13). Epithelial surfaces are complex chemical and biological barriers that prevent the invasion of microbes or other potentially harmful pathogens, but they also harbor many beneficial microorganisms (14).

Our recent *in vitro* study reported that a mixture of fumitory, mallow, wormwood and chamomile possessed a strong ruminal antioxidant capacity with the potential for inducing desirable changes in the gastrointestinal ecosystem during ruminant fermentation (15). Ruminal volatile fatty acids (VFAs) arise mostly from the fermentation of dietary carbohydrates and are absorbed through the ruminal epithelium; we therefore hypothesized that this herbal mixture, together with organic zinc, would affect not only ruminal fermentation and the microbial population but also ruminal histopathology. Our knowledge is also based on our previous findings indicating that a combination of zinc and a special medicinal herbal mixture can positively influence the health of lambs infected with gastrointestinal nematodes (16). We investigated the effects of dietary supplements containing organic zinc and a mixture of medicinal herbs (*Fumaria officinalis* L., *Malva sylvestris* L., *Artemisia absinthium* L., and *Matricaria chamomilla* L.) on ruminal fermentation, the microbial population and the histopathology of the lambs.

MATERIALS AND METHODS

Lambs, Diets, and Experimental Design

The experimental design followed the standards of the European Union for the protection of animals under European Community guidelines (EU Directive 2010/63/EU). The Ethical Committee of the Institute of Animal Physiology of the Centre of Biosciences of the Slovak Academy of Sciences approved the experimental protocol (resolution no. Ro-4065/18-221/3). Twenty-eight castrated male Improved Valachian lambs ∼5 months old with body weights of 22.6 ± 2.94 kg were housed individually in pens for 30 d for acclimatization to feeding with free access to water. The animals were divided into four groups ($n = 7$) based on their live weights. The experimental treatments were as follows: (a) a basal diet (Control) composed of 700 g DM/d meadow hay and 350 g DM/d ground barley; (b) a basal diet enriched with a zinc chelate of amino acids hydrate (Zn, 70 mg Zn/kg of diet/d); (c) a basal diet enriched with a mixture of herbs (Herbs, 100 g DM/d); and (d) a basal diet enriched with a combination of zinc and the mixture of herbs (Zn+Herbs). Aliquots of zinc Availa-Zn 100 EU (Zinpro Corporation, Eden Prairie, USA) were directly mixed with the ground barley. The mixture of herbs (AGROKARPATY, Plavnica, Slovak Republic) contained 33% each of *F. officinalis* (FO), *M. sylvestris* (MS), and *M. chamomilla* (MC) and 1% *A. absinthium* (AA). All experimental groups received diets for 70 d. The lambs were fed twice daily at the same time each day. Before the feeding on the next day, the feed refused by each lamb was sampled and weighed. All samples of refused feed were composited at the end of the study for each lamb and stored at −20°C for later analysis to evaluate nutrient intake. The lambs were weighed at the beginning of the study and on day 35 (D35) and D70. Samples of blood were collected on D0, D35, and D70 from the jugular vein of each lamb using a 21-gauge needle and syringe and deposited into microtubes containing 1.6 mg/mL EDTA-K3 (Sarstedt AG & Co, Nümbrecht, Germany). Hematological parameters were immediately determined using an Abbott CELL-DYN 3700 automated hematological analyzer (Global Medical Instrumentation, Inc., Ramsey, USA).

All animals were killed according to European Commission rules (Council Regulation 1099/2009) for slaughtering procedures (17) at the end of the experiment on three consecutive days (at the abattoir of the Centre of Biosciences of SAS, Institute of Animal Physiology, Košice, Slovakia, No. SK U 06018). The carcasses were sent to the Department of Pathological Anatomy and Pathological Physiology, University of Veterinary Medicine and Pharmacy in Košice, Slovak Republic. Ruminal contents were collected, and samples of fresh ruminal tissues were fixed in 10% neutral buffered formalin.

In vitro Experiment

The experiment was carried out using the *in vitro* gas production technique (IVGPT) on batch-culture incubations of buffered ruminal fluid (RF) incubated at 39°C for 24 h under anaerobic conditions (18). The ruminal contents were collected separately from each lamb of each treatment immediately after the slaughter in the abattoir, packed in prewarmed flasks and transported

TABLE 1 | Chemical compositions (g/kg DM) of the dietary ingredients.

Item	Meadow hay	Barley	Herbs[a]	Availa-Zn100 EU[b]	Barley + Availa-Zn100[c]
Dry matter (DM, g/kg)	894	875	874	976	869
Neutral-detergent fiber	535	277	390	286	516
Acid-detergent fiber	345	108	217	237	138
Nitrogen	23	22	33	34	26
Crude protein	144	137	206	212	163
Ash	77	25	127	361	27

[a]*Dry medicinal herbs (AGROKARPATY, Plavnica, Slovak Republic);* [b]*Availa-Zn 100 EU (Zinpro Corporation, Eden Prairie, USA);* [c]*Zn diet, a mixture of zinc (Availa-Zn 100 EU) and ground barley.*

to the laboratory as was previously described (3). The ruminal contents were forced through four layers of cheesecloth and pooled in equal volumes based on the dietary treatments of the donor animals. The pooled RF was purged with CO_2, mixed with McDougall's buffer (19) in a 1:1 ratio and dispensed in volumes of 35 mL into fermentation bottles (120 mL) containing 250 mg (DM basis) of a substrate. Meadow hay (MH) and barley grain (BG) were used as the basic components of the diet (700:300, w/w) with the use of additive zinc (0.025 g/bottle), herbs (0.025 g/bottle) or both, respectively. Herbs, MH and BG were ground using a grinder (Molina, MIPAM, Ceské Budějovice, Czech Republic) and sieved through 0.15–0.40 mm screens. The *in vitro* experiment was arranged in a completely randomized design using the four diets (Control, Zn, Herbs, and Zn+Herbs) in fermentations with the four inocula of ruminal fluids (Control, Zn, Herbs and Zn+Herbs), with three replicates (three incubation bottles) for each diet and inoculum. The experiment was repeated three times within three consecutive days ($n = 3 \times 3$).

Chemical Analysis and Measurements

The dietary substrates were analyzed in triplicate using standard procedures for dry matter (method no. 967.03), nitrogen (method no. 968.06), crude protein (method no. 990.03) and ash (method no. 942.05) (20). The contents of acid-detergent fiber (ADF) and neutral detergent fiber (NDF) were determined (21) using the FiberCap™ 2021/2023 system (FOSS Analytical AB, Höganäs, Sweden). NDF in the forages was assayed without a thermally stable amylase and was expressed inclusive of residual ash. NDF in the concentrate was assayed using a thermally stable amylase and was expressed inclusive of residual ash. ADF was also expressed inclusive of residual ash. The chemical compositions of the dietary substrates are presented in **Table 1**.

The volume of accumulated total gas was determined after 24 h using IVGPT. For the analysis of methane, 1 mL of gas was collected using IVGPT and an air-tight syringe (GASTIGHT Syringes, Hamilton Bonaduz AG, Switzerland) and injected into a gas chromatograph. The VFAs and methane were analyzed on a PerkinElmer Clarus 500 gas chromatograph (Perkin Elmer, Shelton, USA) (22). Methane production *in vivo* was calculated based on the stoichiometric relationships between VFA composition and methane production (23). The pHs of the batch cultures were measured using a pH meter (InoLab pH Level 1, Weilheim, Germany). The concentrations of ammonia-N in the inocula were determined using the phenol-hypochlorite method (24).

Analysis of Flavonoids and Phenolic Acids

Each of the herbaceous materials (100 mg), *F. officinalis*, *M. sylvestris*, *A. absinthium,* and *M. chamomilla*, were ground to a fine powder and extracted three times with 80% methanol (MeOH) at 40°C for 30 min. The extracts were evaporated to dryness, dissolved in 2 mL of Milli-Q water (acidified with 0.2% formic acid) and purified by solid-phase extraction using a 60-mg Oasis HLB 3cc Vac Cartridge (Waters Corp., Milford, USA). The Milli-Q water was prepared by an ultrapure water system (Barnstead International, Dubuque, USA). The cartridges were washed with 0.5% MeOH to remove carbohydrates and then washed with 80% MeOH to elute the phenolics. The phenolic fraction was re-evaporated and dissolved in 1 mL of 80% MeOH (acidified with 0.1% formic acid). The sample was then centrifuged at 23,000 × g for 5 min before spectrometric analysis. All analyses were performed in triplicate for three independent samples and stored at −20°C before analysis.

Analysis of Alkaloids

Herbal materials from FO were ground to a fine powder, and 100 mg were extracted with 0.5M H_2SO_4 in an ultra-bath at 25°C for 20 min; the procedure was then repeated, and the filtrates were combined. The filtrates were adjusted to pH 9-10 using 1M NaOH and separated using $CHCl_3$. The lower organic layer was collected, evaporated to dryness under reduced pressure and then dissolved in 80% MeOH for further analysis.

Ultra-High-Resolution Mass Spectrometry (UHRMS)

The bioactive compounds of each medicinal herb (FO, MS, AA and MC) were identified using UHRMS on a Dionex UltiMate 3000RS system (Thermo Scientific, Darmstadt, Germany) with a charged aerosol detector connected to a Compact high-resolution quadrupole time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The metabolome of the mixture of herbs was chromatographically separated on a 2.1 × 100 mm, 2.6 μm, Kinetex C18 column (Phenomenex, Torrance, USA), with mobile phase A consisting of 0.1% (v/v) formic acid (FA) in water and mobile phase B consisting of 0.1% (v/v) FA in acetonitrile. A linear gradient from 7 to 30% of phase B in phase A over 20 min was used to separate the phenolic compounds, with a short 0.3 min calibration segment from 0 to 0.5 min. The flow rate was 0.3 mL/min, and the column was held at 25°C. Spectra were acquired in negative-ion mode over a mass range from m/z 100 to 1,500 with a frequency of 5 Hz. The operating parameters of the ESI ion source were: capillary voltage, 3 kV; dry gas flow, 6 L/min; dry gas temperature, 200 °C; nebulizer pressure, 0.7 bar; collision radio frequency, 700.0 V; transfer time, 100.0 μs and pre-pulse storage, 7.0 μs. Ultrapure nitrogen was used as the drying and nebulizer gas, and argon was used as the collision gas. The collision energy

was set automatically from 15 to 75 eV depending on the mass of the fragmented ion. The data were calibrated internally using sodium formate introduced into the ion source at the beginning of each separation via a 20-μL loop. The spectra were processed using Bruker DataAnalysis 4.3 software (Bruker Daltonik GmbH, Bremen, Germany). The amounts of the phenolic acids in the samples were calculated as the chlorogenic acid (CAS 327-97-9, 3-caffeoylquinic acid) equivalent, and hyperoside (CAS 482-36-0, quercetin 3-galactoside) was used for calculating the amounts of the flavonoids identified. Stock solutions of hyperoside and chlorogenic acid were prepared in MeOH at concentrations of 3.1 and 4.1 mg/mL, respectively, and kept frozen until used. Calibration curves for these two compounds were constructed based on seven concentration points (from 500 to 3.9 μg/mL).

The total content of alkaloids was determined as the chelidonine (CAS 476-32-4) equivalent from the calibration curves based on seven concentration points of chelidonine (from 200 to 1.2 μg/mL). The alkaloids were separated using the same HPLC conditions as with the phenolic compounds, with one exception: positive-ion mode was used for the acquired spectra in auto MS/MS. All analyses were performed in triplicate.

Quantification of Ruminal Microbes

Samples for counting ciliate protozoa were fixed in equal volumes of 8% formaldehyde, and the protozoa were counted and identified microscopically as described by Williams and Coleman (25). DNA for quantifying bacteria was isolated from the ruminal samples using a Mini Bead-Beater (BioSpec, Bartlesville, USA) for cell lysis (26), followed by purification using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). DNA concentrations and qualities were measured using a NanoPhotometer® NP80 (Implen GmbH, München, Germany). Eubacteria and archaea were quantified by real-time PCR using the PCR primers (27). The relative abundance of the 16S rRNA gene was expressed as an arbitrary unit (AU) relative to the total abundance of bacterial genes of the Control group.

Histological Parameters

Samples of fresh ruminal tissues were washed in a phosphate buffer (0.1 M, pH 7.4), put in plastic containers and fixed in a 10% buffered FA solution as pieces of tissue spread on flat polystyrene. The fixed material was processed using a series of reagents and embedded in Paraplast PLUS paraffin blocks (Leica, Buffalo Grove, USA), which were then cut with a rotary microtome into sections 3.5 μm thick. Slides with a paraffin section were automatically stained with hematoxylin and eosin (Varistain Gemini Thermo Scientific, Runcorn, UK). An Axio Lab. 1 microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss Axiocam ERc5s digital camera was used for histological evaluation. Photographs were analyzed and recorded using ZEN 2.3 (blue edition) software (Carl Zeiss Microscopy GmbH, 2011).

Data Analysis

The data were statistically analyzed using GraphPad Prism 8.3.0 (538) 2019 (GraphPad Software, Inc., San Diego, USA). Data for the parameters of ruminal fermentation and *in vitro* ciliate populations were analyzed using two-way analyses of variance (ANOVAs). The model included effects for diets, inocula and the diet × inoculum interaction. Statistical analysis of the hematological parameters used an ANOVA as a repeated-measure mixed model that represented the four animal groups and the sampling days. *In vivo* data were evaluated by multiple comparisons of one-way ANOVAs using Dunnett's multiple comparisons test. The total and differential counts of the ruminal ciliates were analyzed using the non-parametric Kruskal-Wallis test. The effects were determined to be significant at $P < 0.05$.

RESULTS

Phytochemical Substances in the Medicinal Herbs

The phytochemical substances in FO consisted of 12.211 g/kg DM flavonoids, 3.961 g/kg DM phenolic acids (**Table 2**) and 6.015 g/kg DM alkaloids (**Table 3**). The phytochemical substances in MS consisted of 6.479 g/kg DM flavonoids and 0.654 g/kg DM phenolic acids (**Table 2**). The phytochemical substances in AA consisted of 0.349 g/kg DM flavonoids and 6.482 g/kg DM phenolic acids (**Table 2**). The phytochemical substances in MC consisted of 2.442 g/kg DM flavonoids and 12.084 g/kg DM phenolic acids (**Table 2**).

Effect of Dietary Substrates on Ruminal Fermentation *in vitro*

The effects of the dietary substrates and inocula on the parameters of ruminal fermentation *in vitro* are presented in **Table 4**. The inocula of the donor animals affected the values of all parameters of fermentation ($P < 0.001$). Diet significantly affected total gas ($P < 0.046$) and methane ($P < 0.001$) production. The amount of *n*-butyrate also varied among the dietary treatments ($P < 0.001$), with *n*-butyrate concentrations higher for the Zn and Zn+Herbs groups than the Control group. The different ruminal inocula also significantly affected the protozoal populations of the lambs *in vitro* (**Table 5**).

Effects of Zinc and Herbal Diets on Ruminal Fermentation and Microbiota in the Lambs

The dietary supplements did not significantly affect ($P > 0.05$) the parameters of ruminal fermentation in the lambs (**Table 6**). The number of ruminal ciliated protozoa, expressed as counts per gram of wet ruminal content or as counts per gram of dry ruminal content in the lambs did not differ significantly ($P > 0.05$) between the groups (**Table 7**). The lambs contained three ciliate populations. Nineteen animals (68%) had mixed A-B type populations, consisting of *Polyplastron multivesiculatum* (100% prevalence), *Epidinium ecaudatum caudatum* (84% prevalence), and *Ophryoscolex caudatus tricoronatus* (53% prevalence), seven animals (25%) had A type populations, consisting of *P. multivesiculatum* (100% prevalence) and *O. caudatus tricoronatus* (29% prevalence), and two animals (7%) had B type populations, consisting of *E. ecaudatum caudatum* (100% prevalence)

TABLE 2 | Concentrations of the main bioactive compounds in medicinal herbs (g/kg DM).

RT (min)	UV (nm)	*m/z*[M-H]$^-$	MS^2	MS^2 fragments	Formula	Compounds	Flavonoids	Phenolic acids
Fumaria officinalis								
7.00	250/326	295.046	179.0338		$C_{13}H_{12}O_8$	Caffeoylmalic acid		1.212
7.80	227/315	163.0395	119.0499		$C_9H_8O_3$	O-Coumaric acid		0.742
9.00	255/352	625.1398	301.0337		$C_{27}H_{30}O_{17}$	Quercetin-O-Hex-Hex	2.384	
9.40	255/352	595.1287	301.0339		$C_{26}H_{28}O_{16}$	Quercetin O-Pen-Hex	3.500	
9.90	252/351	609.1472	300.0279	285	$C_{27}H_{30}O_{16}$	Isoquercitrin O-Dhex		0.934
10.20	255/354	463.0882	301.0337		$C_{21}H_{20}O_{12}$	Quercetin O-Hex	1.706	
10.90	221/329	593.1520	285.0397		$C_{27}H_{30}O_{15}$	Kaempferol-3-O-rutinoside	0.464	
11.50	255/365	639.1561	315.0504		$C_{28}H_{32}O_{17}$	Isorhamnetin-O-Hex-Hex	0.558	
						Total contents:	12.211	3.961
Malva sylvestris								
7.00	523	757.1846	347.0761	329,261,509	$C_{32}H_{39}O_{21}$	Delphinidin 5-glucoside 3-lathyroside	1.644	
7.90	308	163.0381	119.0502		$C_9H_8O_3$	Coumarinic acid		0.468
10.00		609.1458	301.0330		$C_{27}H_{31}O_{16}$	Quercetin-3-O-rutinoside	0.395	
10.20	268/343	447.0928	285.0386		$C_{21}H_{20}O_{11}$	Kaempferol-O-Hex	0.494	
11.40	268/336	431.0978	269.0435		$C_{21}H_{20}O_{10}$	Apigenin-O-Hex	1.560	
						Total contents:	6.479	0.654
Artemisia absinthium								
4.10	215/325	353.0877	191.0567	179,161,135	$C_{16}H_{18}O_9$	Chlorogenic acid		3.416
11.00		515.1193	353.0867	191,179,135	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid		2.124
11.20		653.1719	345.0595	330,302	$C_{29}H_{34}O_{17}$	Spinacetin 3-rutinoside	0.241	
11.70		515.1192	353.0869	173,179,191,155	$C_{25}H_{24}O_{12}$	4.5-Dicaffeoylquinic acid		0.610
						Total contents:	0.349	6.482
Matricaria chamomilla								
4.30	215/300	353.0877	191.0567		$C_{16}H_{18}O_9$	3-O-Caffeoylquinic acid		1.777
9.00	235/290/319	355.1029	193.049	149	$C_{16}H_{20}O_9$	Methyl 4-O-beta-d-glucopyranosyl caffeate		3.202
9.70	255/354	463.0879	301.0337	151	$C_{21}H_{20}O_{12}$	Quercetin O-Hex	0.199	
10.30	257	447.0920	285.0386		$C_{21}H_{20}O_{11}$	Kaempferol O-Hex	1.363	
10.70	217/291/325	515.1189	353.0877	179,191	$C_{25}H_{24}O_{12}$	3.5-Dicaffeoylquinic acid		0.824
11.00	217/291/325	515.1197	353.0869	191,179	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid		3.016
11.40	266/300	431.0976	269.0434		$C_{21}H_{20}O_{10}$	Apigenin O-Hex	0.150	
11.70	215/290/325	515.119	353.0868	173,179,191	$C_{25}H_{24}O_{12}$	4.5-Dicaffeoylquinic acid		0.851
14.40	218/268/339	473.1085	269.0427	406	$C_{23}H_{22}O_{11}$	Apigenin -O-(Hex-Ac)	0.210	
						Total contents:	2.442	12.084

but no *Polyplastron* or *Ophryoscolex*. All animals had *Dasytricha ruminantium*, *Isotricha intestinalis*, *I. prostoma,* and *Entodinium* spp.

The total bacterial populations (**Figure 1A**) were significantly lower ($P < 0.05$) for the Zn and Zn+Herbs groups than the Control group, but the relative abundances of *Ruminococcus albus* (**Figure 1C**), *Streptococcus bovis* (**Figure 1D**) and *Butyrivibrio proteoclasticus* (**Figure 1E**) were significantly higher in the group fed the Zn diets. In contrast, the relative abundance of *Ruminococcus flavefaciens* (**Figure 1K**) was significantly lower ($P < 0.05$) in the Zn than the Control group. The relative abundance of *Fibrobacter succinogenes* (**Figure 1F**) was significantly lower ($P < 0.05$) in the Herbs than the Control group. The other microbial populations, such as those of *Archaea* (**Figure 1B**), *Butyrivibrio fibrisolvens* (**Figure 1G**), *Prevotella* (**Figure 1H**), Clostridium aminophilum (**Figure 1I**) and Megasphaera elsdenii (**Figure 1J**), did not differ significantly ($P > 0.05$) among the groups.

Hematological Parameters

The count of red blood cells, hemoglobin level and hematocrit were not significantly influenced by time, treatment or the treatment × time interaction ($P > 0.05$) (**Table 8**). Time significantly affected the mean corpuscular volumes ($P < 0.001$). Treatment and time significantly affected neutrophil levels ($P < 0.05$), and time significantly affected the counts of lymphocytes and eosinophils ($P < 0.001$ and < 0.05, respectively).

Effects of Diet on the Morphological Parameters of the Ruminal Papillae

The histology of the ruminal papillae of the Control and Zn groups were normal (**Figures 2A,B**). The sizes of the papillae

TABLE 3 | Concentrations of the main alkaloids in *Fumaria officinalis* (g/kg DM).

RT (min)	UV (nm)	*m/z*[M-H]$^-$	MS^2	MS^2 fragments	Formula	Compounds	Alkaloids
7.70	272	354.1366	305.0811	279,233,323,336	$C_{20}H_{19}NO_5$	Parfumine	0.884
8.40	280	328.1572	265.0865	237,297,313,178	$C_{19}H_{21}NO_4$	Cularimine	0.102
8.50	288	370.1678	291.1029	263,352,337	$C_{21}H_{23}NO_5$	Fumaricine	0.102
8.90	285	326.1410	311.1172	277,294,251,178	$C_{19}H_{19}NO_4$	Cheilanthifoline	0.231
9.20	286	354.1360	275.0713	336,247	$C_{20}H_{19}NO_5$	Chelidonine	0.154
9.40	289	398.1621	338.1397	277,323,249	$C_{22}H_{23}NO_6$	Not determined	0.530
10.20	289	354.1362	275.0692	247,293,206	$C_{20}H_{19}NO_5$	Protopine	0.873
10.60	288	354.1727	206.1139	275,311,338,292	$C_{21}H_{23}NO_4$	Protopine type	0.367
10.80	271	352.1193	279.0647	309,321,263,251	$C_{20}H_{17}NO_5$	Fumariline	1.728
11.20	288	324.1230	249.0764	307,277,219,176	$C_{19}H_{17}NO_4$	Stylopine	0.785
						Total contents:	6.015

m/z, mass-to-charge ratio.

TABLE 4 | Effect of ruminal inocula and diets on the fermentation parameters *in vitro* ($N = 9$).

Inoculum	Diet	pH	NH_3-N (mg/L)	Gas (mL/g)	CH_4 (mM)	IVDMD (g/kg DM)	VFA mM	A mol%	P mol%	iB mol%	nB mol%	iV mol%	nV mol%	nC mol%
Control	Control	6.84	217	229	2.74	574	46.7	66.8	14.9	0.97	13.9	1.81	1.38	0.28
	Zn	6.92	192	222	2.80	518	43.9	66.5	15.2	0.84	14.3	1.60	1.37	0.25
	Herbs	6.91	214	240	2.88	581	46.6	67.0	15.0	0.85	13.8	1.71	1.36	0.31
	Zn+Herbs	6.87	198	227	2.97	502	46.3	66.8	15.3	0.73	14.0	1.54	1.42	0.33
Zn	Control	6.96	305	233	3.06	341	43.8	65.2	15.3	1.55	13.0	2.79	1.75	0.40
	Zn	6.96	301	218	2.62	325	41.1	64.5	15.6	1.55	13.6	2.70	1.74	0.37
	Herbs	6.91	330	250	3.61	347	45.6	65.3	15.5	1.57	12.8	2.73	1.77	0.34
	Zn+Herbs	6.82	273	231	3.31	306	44.0	65.3	15.8	1.27	13.2	2.41	1.69	0.34
Herbs	Control	6.99	248	218	2.66	525	36.2	66.9	16.1	1.10	11.9	2.16	1.59	0.25
	Zn	6.98	214	198	1.73	492	37.8	65.8	15.9	1.08	13.1	2.11	1.65	0.28
	Herbs	6.95	222	216	2.66	554	41.5	66.8	16.4	1.07	11.9	2.05	1.55	0.26
	Zn+Herbs	6.98	216	211	2.47	515	39.5	66.3	16.2	1.05	12.7	1.95	1.58	0.29
Zn+Herbs	Control	7.06	182	184	2.68	585	35.0	65.7	16.7	1.11	12.8	1.95	1.54	0.22
	Zn	7.05	184	173	2.28	575	32.5	64.7	16.3	1.07	14.1	1.94	1.66	0.23
	Herbs	7.04	204	178	2.76	622	35.9	65.8	16.6	1.08	12.9	1.93	1.57	0.21
	Zn+Herbs	7.06	197	180	2.52	571	34.1	65.0	16.7	1.01	13.6	1.83	1.69	0.23
SEM		0.037	24.4	8.65	0.186	39.7	2.60	0.564	0.364	0.104	0.289	0.199	0.065	0.028
Significance of the effects:														
Diet		0.512	0.606	0.046	0.001	0.316	0.256	0.159	0.857	0.211	0.001	0.478	0.771	0.730
Inoculum		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Diet × inoculum		0.457	0.985	0.942	0.299	0.996	0.999	0.999	0.997	0.983	0.877	0.999	0.877	0.600

IVDMD, In vitro dry matter digestibility; VFA, Volatile fatty acids; A, Acetate; P, Propionate; iB, Iso-butyrate; nB, N-butyrate; iV, Iso-valerate; nV, N-valerate; nC, N-caproate.

varied in the Herbs group (**Figure 2C**), and both the type and number of keratinized epithelial cells varied in the Zn+Herbs group (**Figure 2D**). A focally mixed infiltration of inflammatory cells in individual papillae within the epithelial layer was observed in two animals fed the Herbs diet and within the lamina propria in most animals fed the Zn+Herbs diet.

DISCUSSION

Our previous results indicated that the addition of zinc to the diet of lambs did not negatively influence microbial activity in the rumen or large intestine (4). A mixture of herbs containing FO, MS, AA, and MC also possessed strong ruminal antioxidant capacity and reduced gastrointestinal concentrations of methane and ammonia *in vitro* (15). To the best of our knowledge, however, the present experiment is the first to determine the interactions between supplementations with organic zinc and herbs by combining phytochemical, physiological, microbiological and histopathological measurements in the rumen of lambs.

The phytochemical substances in FO consisted of flavonoids, alkaloids and phenolic acids. There were mainly quercetin-O-Hex-Hex (2.384 g/kg DM), quercetin O-Pen-Hex (3.5 g/kg DM), fumariline (1.728 g/kg DM), fumaricine (0.102 g/kg DM), and caffeoylmalic acid (1.212 g/kg DM). The flavonoid (flavonol) quercetin possesses various antioxidative and anti-inflammatory

TABLE 5 | Effect of ruminal inocula and diets on the protozoal population *in vitro* ($N = 9$).

Inoculum	Diet	*Dasytricha ruminantium* (n/mL)	*Isotricha* spp. (n/mL)	*Polyplastron multivesiculatum* (n/mL)	*Entodinium* spp. (10^3 /mL)	Total protozoa (10^3 /mL)
Control	Control	850	1,010	330	368	378
	Zn	1,320	1,140	350	336	347
	Herbs	1,450	1,230	480	329	338
	Zn+Herbs	2,510	1,280	570	334	343
Zn	Control	2,290	1,880	580	374	380
	Zn	2,330	2,020	710	401	407
	Herbs	2,430	1,830	710	425	430
	Zn+Herbs	2,750	1,900	690	395	401
Herbs	Control	3,270	800	310	321	326
	Zn	3,730	640	250	292	297
	Herbs	3,204	530	210	314	318
	Zn+Herbs	3,890	520	260	292	297
Zn+Herbs	Control	2,440	1,120	180	313	318
	Zn	2,610	1,620	140	350	357
	Herbs	2,060	1,040	130	327	332
	Zn+Herbs	2,720	1,670	250	329	335
SEM		111.5	81.8	29.0	9.1	9.2
Significance of the effects:						
Diet		0.073	0.734	0.563	0.982	0.982
Inoculum		< 0.001	< 0.001	< 0.001	0.004	0.004
Diet × inoculum		0.799	0.933	0.870	0.979	0.982

TABLE 6 | Effect of zinc and herbs on the parameters of ruminal fermentation in the lambs ($N = 7$).

Item	Control	Zn	Herbs	Zn+Herbs	SD	*P*-value
pH	6.84	6.95	6.89	7.05	0.245	0.468
Ammonia (mg/L)	110	129	121	102	38.5	0.629
Methane (mM)	0.376	0.382	0.378	0.381	0.071	0.832
Total VFA (mM)	53.9	42.6	47.2	37.3	13.9	0.191
Acetate (mol%)	69.1	69.1	69.8	68.3	2.04	0.663
Propionate (mol%)	13.8	13.9	14.5	14.5	1.55	0.753
n-Butyrate (mol%)	13.5	12.1	11.8	12.5	1.59	0.240
iso-Butyrate (mol%)	1.03	1.73	1.10	1.65	0.879	0.356
n-Valerate (mol%)	0.914	0.910	0.936	1.03	0.221	0.717
iso-Valerate (mol%)	1.33	2.00	1.51	1.77	0.822	0.478
n-Caproate (mol%)	0.300	0.267	0.263	0.204	0.119	0.546
A:P	5.10	5.04	4.83	4.77	0.567	0.673

TABLE 7 | Effects of zinc and herbs on the number of ruminal ciliated protozoa in the lambs ($N = 7$).

Genus/treatment	Control	Zn	Herbs	Zn+Herbs	SD	*P*-value
Dasytricha sp. (c/g wRC)[a]	4,754	5, 28	6,717	8,619	5,004	0.433
Isotricha spp. (c/g wRC)	2,280	3,132	2,656	2,043	1,581	0.417
Polyplastron spp. (c/g wRC)	2,571	2,723	2,311	663	1,445	0.064
Epidinium spp. (c/g wRC)	15,905	160	3,055	13,612	9,558	0.662
Entodinium spp. (c/g wRC)	465,316	485,455	412,898	457,362	93,530	0.392
Total protozoa (c/g wRC)	489,413	500,892	427,987	483,593	95,191	0.454
Dasytricha sp. (c/g DM)[b]	492	609	701	904	503	0.529
Isotricha spp. (c/g DM)	301	401	327	241	228	0.275
Polyplastron spp. (c/g DM)	234	333	291	75	216	0.133
Epidinium spp. (c/g DM)	2,258	18	274	1,566	1,123	0.044
Entodinium spp. (c/g DM)	63,146	59,933	49,355	52,791	17,298	0.490
Total protozoa (c/g DM)	66,430	61,914	50,987	55,729	17,936	0.400

[a] *Expressed as count (c) per gram of wet ruminal content (wRC);* [b] *Expressed as count per gram of dry matter (DM) of ruminal content.*

effects and metabolic health-promoting properties (28). Both isoquinoline alkaloids fumariline and fumaricine contribute to the important pharmacological activities of FO (29). The phenolic compound caffeoylmalic acid can protect protein from degradation in ruminants that use forage protein (30). The concentrations of the flavonoids, delphinidin 5-glucoside 3-lathyroside (1.644 g/kg DM) and apigenin-O-Hex (1.56 g/kg DM), were highest in MS. The health-promoting effect of apigenin O-Hex, which has therapeutic potential, has been reported (31), but flavonoids generally possess beneficial biochemical properties with predominantly protective roles against many diseases (32). The phytochemical substances in AA consisted mainly of phenolic acids, including chlorogenic acid (3.416 g/kg DM) and 1,5-dicaffeoylquinic acid (2.124 g/kg DM), which possess antibacterial, anthelmintic, anti-inflammatory and antioxidant biological activities *in vitro* and *in vivo* (33, 34).

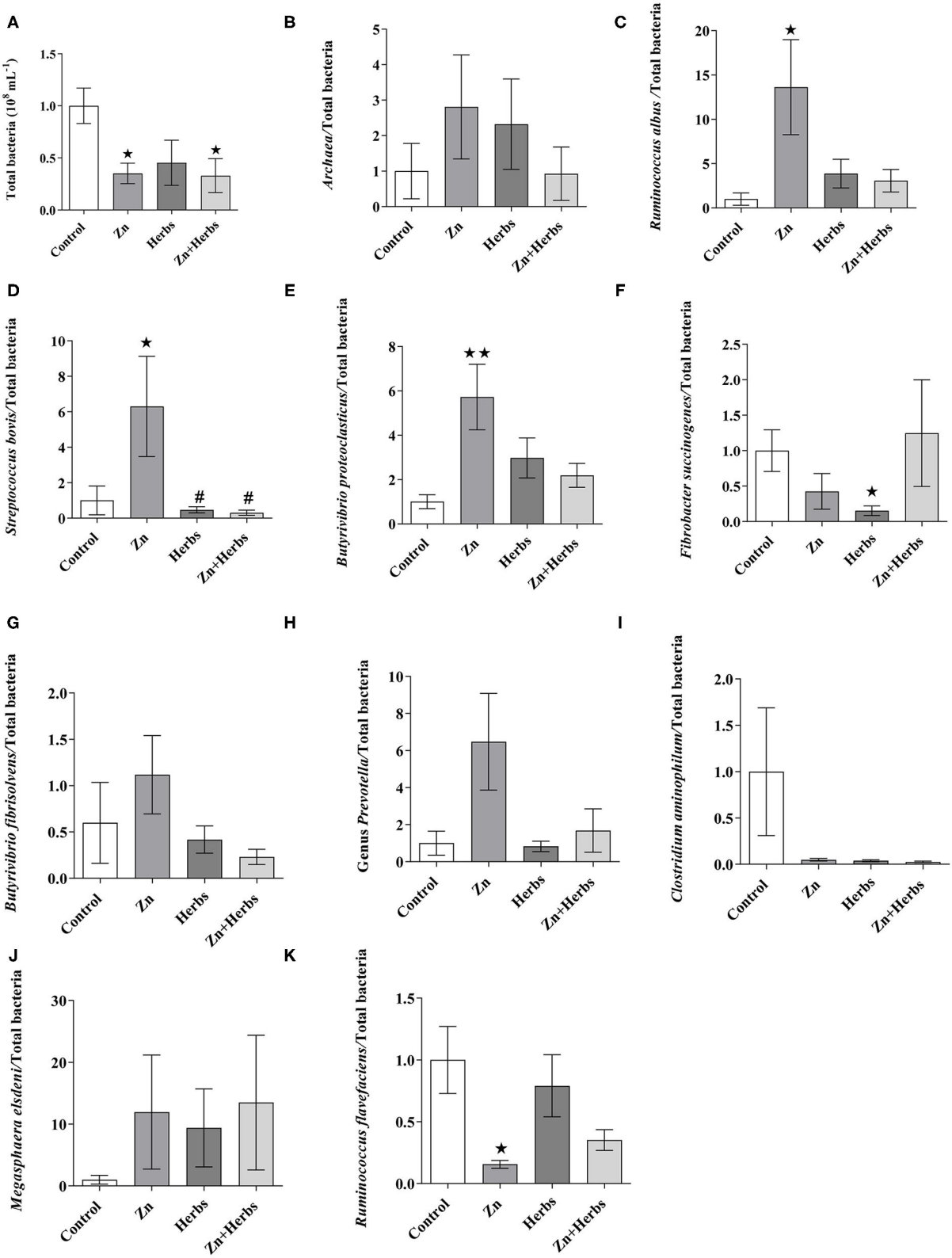

FIGURE 1 | Effects of the Control, Zn, Herbs, and Zn+Herbs diets on the relative abundance of the 16S rRNA gene (expressed relative to the total abundance of bacterial genes in the Control group) of the ruminal bacterial population for **(A)** total bacteria, **(B)** Archaea, **(C)** *Ruminococcus albus*, **(D**) *Streptococcus bovis*, **(E)** *Butyrivibrio proteoclasticus*, **(F)** *Fibrobacter succinogenes*, **(G)** *Butyrivibrio fibrisolvens*, **(H)** *Prevotella,* **(I)** Clostridium *aminophilum*, **(J)** Megasphaera *elsdeni,* and **(K)** *Ruminococcus flavefaciens*. $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to the Control group; $^{\#}P < 0.05$ relative to the Zn group.

TABLE 8 | Effects of zinc and herbs on the hematological parameters of the lambs ($N = 7$).

Item	Day	Control	Zn	Herbs	Zn+Herbs	SD	Significance of effects:		
							Treatment	Time	Treatment × time
Red blood cells (T/L)	0	11.1	10.8	10.8	11.0	0.324			
	35	10.5	10.5	10.7	10.4	0.863	NS	NS	NS
	70	10.1	10.8	10.4	9.47	1.28			
Hemoglobin (g/L)	0	102.8	99.1	96.2	98.0	7.08			
	35	97.8	99.2	101.3	98.6	7.56	NS	NS	NS
	70	82.7	100.0	100.6	98.9	15.8			
Hematocrit (L/L)	0	0.194	0.218	0.214	0.216	0.028			
	35	0.228	0.230	0.229	0.222	0.014	NS	NS	NS
	70	0.226	0.236	0.229	0.208	0.025			
Mean corpuscular volume (fL)	0	20.0	20.3	19.9	19.8	1.15			
	35	21.8	21.8	21.6	21.5	1.28	NS	***	NS
	70	22.6	22.0	22.0	22.1	1.14			
Total leukocytes (g/L)	0	8.26	8.70	7.67	6.60	2.34			
	35	8.33	8.45	8.25	7.70	1.69	NS	NS	NS
	70	8.55	6.54	8.83	8.59	1.33			
Neutrophils (g/L)	0	2.60	3.90	2.56	2.09	1.36			
	35	2.79	3.00	3.03	2.37	0.897	NS	NS	*
	70	3.11	2.01	4.04	3.26	0.953			
Lymphocytes (g/L)	0	3.14	2.50	2.44	2.37	0.903			
	35	3.54	3.51	3.27	3.68	1.41	NS	*	NS
	70	2.96	3.00	2.76	3.03	0.894			
Monocytes (g/L)	0	2.29	1.76	2.41	1.72	0.715			
	35	1.75	1.53	1.61	1.39	0.667	NS	NS	NS
	70	2.18	1.20	1.77	1.94	0.766			
Eosinophils (g/L)	0	0.052	0.044	0.036	0.042	0.019			
	35	0.084	0.150	0.112	0.120	0.079	NS	***	NS
	70	0.056	0.098	0.065	0.092	0.051			
Basophils (g/L)	0	0.257	0.499	0.228	0.370	0.396			
	35	0.170	0.258	0.074	0.132	0.154	NS	NS	NS
	70	0.239	0.221	0.199	0.267	0.207			

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Similarly, MC contained phenolic acids (12.084 g/kg DM), mainly methyl 4-O-beta-d-glucopyranosylcaffeate (3.202 g/kg DM), with well-known antioxidant activity (35), and contained derivatives of caffeoylquinic acid, which have anti-inflammatory biological activities (36).

The mixture of the dry medicinal herbs had high concentrations of flavonoids, especially quercetin (7.6 mg/g DM) (15). Quercetin, after intraruminal application (10 and 50 mg/kg BW) in cows, is a flavonoid extensively degraded by ruminal microbiota without negative effects on ruminal fermentation (37). The administration of some flavonoids with antimicrobial properties, however, can affect the gastrointestinal microbiota. The Zn, Herbs and Zn+Herbs diets under *in vitro* conditions in our study only affected the levels of total gas, methane and *n*-butyrate. The reported effects of flavonoids as potential dietary additives for ruminants (e.g., quercetin, myricetin, kaempferol, and rutin) have been inconsistent, due mainly to their potential antimicrobial effects (38–40).

The *in vitro* ruminal inocula (**Table 5**), however, significantly affected all parameters of fermentation and species of protozoa. The effects of the inocula could be ascribed to the diverse ruminal ciliate populations and companion bacterial populations in the animals. **Table 5** presents the number of protozoa in each group. Not all treatment groups, however, contained *Ophryoscolex* and *Epidinium*. The mixed A-B type ciliate population was also prevalent (19 animals). This finding probably indicates a gradual change from the B type population to the dominant A type population in the lambs, because *Polyplastron* feeds on *Epidinium* until it disappears from the ciliate population (25). The inocula had significant effects, mainly on *Dasytricha* species, probably caused by the long-term dietary supplementation with zinc or herbs, which can influence the composition of the eubacterial community and the enzymatic activities of ruminal microorganisms, especially amylolytic and cellulolytic enzymes (41, 42).

The Zn, Herbs and Zn+Herbs diets did not significantly affect the parameters of fermentation or the protozoal populations in

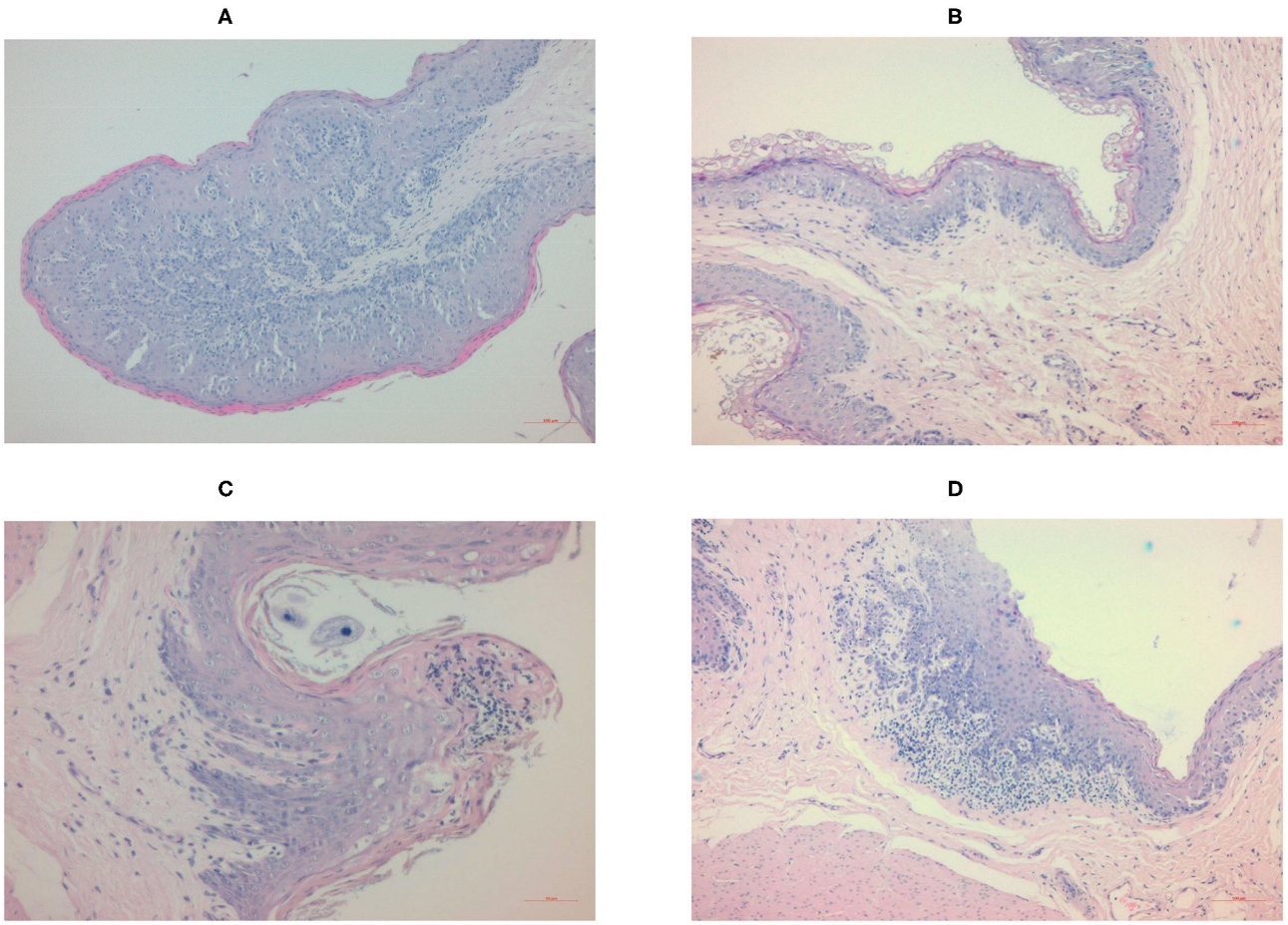

FIGURE 2 | Histology of the ruminal papillae of the lambs in the **(A)** Control, **(B)** Zn, **(C)** Herbs, and **(D)** Zn+Herbs groups. The papillae are stained with hematoxylin-eosin and viewed using a 10 × objective lens.

the lambs. This finding probably indicates relatively low contents of antimethanogenic phytochemical substances or the adaptation of the microbiota to both herbal (43, 44) and/or zinc (45) diets. Herbal diets can influence the ruminal microbiome, the kinetics of fermentation and the response and adaptation to antimethanogenic compounds and diets that sometimes lead to the inconsistent efficacies of phytochemical substances (46). Zn is also involved in a wide assortment of physiological processes, so nutrient digestibility may be affected by supplemental Zn, which is incorporated into enzymes throughout the body and is critical for most metabolic processes in ruminants (47). Zinc in the diet of ruminants can substantially influence ruminal fermentation (48, 49). Low doses of zinc (20–70 mg Zn/kg diet) only weakly affect ruminal fermentation (4, 10), but higher doses (250–1,142 mg Zn/kg diet) can affect ruminal protozoal populations and protein degradation (50). The beneficial effects of zinc (i.e., its antioxidant, anti-inflammatory and antiapoptotic properties) strongly depend on both the source and concentration of the zinc, even though zinc retention in lambs can be similar regardless of these factors (47), and too much or too little zinc in diets can have the opposite effect (51, 52).

The amount of starch in all our diets was similar, so the diets probably similarly influenced the efficiency of the growth of the majority of the ruminal ciliates (53). Total bacteria, however, were lower in the Zn and Zn+Herbs groups than the Control group. The relative abundances of the cellulolytic bacterium *R. albus*, the amylolytic bacterium *S. bovis* and the polysaccharide-degrading bacterium *B. proteoclasticus* were higher, and the abundance of the cellulolytic bacterium *R.* flavefaciens was lower in the Zn group than the other groups. Some bacterial species were probably enriched by Zn supplementation at the expense of total bacterial abundance (45). These changes in total bacteria and the relative abundance of some bacteria in the Zn group were not accompanied by changes in VFAs in ruminal fermentation *in vivo*. This finding indicates a direct effect on ruminal microbiota due to an interaction with crude protein rather than to the benefits of Zn supplementation, which is exerted solely on the host organism (8). The lower relative abundance of cellulolytic bacteria (i.e., *F. succinogenes* and *R. flavefaciens*) in the Zn or Zn+Herbs groups, respectively, however, probably also lowered the digestibility of the substrate *in vitro* in these groups (**Table 4**). The ruminal microbiota may have a specific requirement for zinc

supplementation that does not cause a major shift in the ruminal bacterial community and does not have negative consequences for digestion or animal health (45). Some bacterial phylotypes can also contribute to differences in feed efficiency and host productivity and can or need not depend on the diet (54). The relative abundance of the starch-fermenting bacterium *S. bovis* was lower in the Herbs and Zn+Herbs groups than the Zn group, but the relative abundance of the cellulolytic bacterium *F. succinogenes* was lower in the Herbs and Zn+Herbs groups than the Control group, probably due to the antimicrobial activity of some flavonoids, which can increase competition among bacteria (40, 55).

Ruminal VFAs are absorbed through the ruminal epithelium, and the rate of absorption depends on the VFA concentration, the surface area of the ruminal papillae and the availability of transport proteins (56, 57). The ruminal papillae of the lambs in the Control and Zn groups were histologically normal, with diverse sizes of the papillae and the type and number of keratinized epithelial cells. The external layer of vesiculated keratinized cells of the ruminal epithelium is an absorption barrier to the transport of molecules from the rumen to the blood (58). The stratum corneum in the Zn group contained several layers of vacuolated horn cells, with a large amount of keratin in the cytoplasm and cellular organelles. The lambs in the Herbs and Zn+Herbs groups had ruminal papillae with diverse histological structures, mostly the size of the papillae and type and number of keratinized epithelial cells. Butyrate stimulates the development of ruminal papillae (59), but the *in vitro* molar proportion of *n*-butyrate was higher only in the Zn and Zn+Herbs groups. The amount of ruminal VFA absorption can decrease as ruminal parakeratosis increases (60), and a physical barrier could reduce the transport of VFAs to the deepest layers of the epithelium. The health of the ruminal epithelium probably deteriorated because the infiltration of inflammatory cells in individual papillae in the epithelial layer and the lamina propria was focally mixed in the lambs in the Herbs and Zn+Herbs groups, respectively. The hematological parameters of the lambs, however, were not affected by the treatments. The application of the Herbs treatment for 70 d may have been too long.

Nutraceuticals provide health benefits beyond basic nutrition. The vast number of naturally occurring health-enhancing substances are of herbal origin, but many physiologically active components, such as trace elements, also play important roles in the promotion of animal health. Limited information is available on the effects of nutraceuticals such as zinc and/or herbs on blood profiles (16, 61). Pharmacological and clinical studies suggest that *M. sylvestris*, *A. absinthium* and *M. chamomilla* are promising herbs for the treatment of gastrointestinal disorders (62). Dietary supplementation with *A. absinthium* can also enhance the rate of growth of lambs, thereby increasing weight gains (63). Different nutrients, however, may generally improve the absorptive capability of the ruminal epithelium, protect the epithelium against damage and alter the expression of genes regulating ruminal epithelial morphology (64–66).

CONCLUSIONS

The ability of dietary supplementation with organic zinc (70 mg Zn/kg diet) and herbs (100 g DM/d) to influence ruminal fermentation and the composition of ruminal microbiota *in vitro* was not confirmed *in vivo*. The dietary supplements did not significantly affect the parameters of ruminal fermentation or the protozoal population of the lambs, probably because the lambs adapted to the diets during the 70-d feeding, with lower total bacteria and a shift in the relative abundances of cellulolytic and amylolytic bacteria in the Zn group. Our results, however, also indicated that long-term dietary supplementation with organic zinc combined with a mixture of medicinal herbs could negatively affect the health of the ruminal epithelium. More *in vivo* experiments are therefore necessary.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of the Institute of Animal Physiology of Centre of Biosciences of SAS approved the experimental protocol (resolution number Ro-3355/16-221).

AUTHOR CONTRIBUTIONS

DP, DM, and KK: resources, formal analysis, and investigation. PK, HH, and DM: investigation, methodology, and software. SK: investigation and methodology. AL: histological analyses. SS: bioactive compound analysis. AC and MS-S: conceptualization and data curation. KC: project administration, funding acquisition, and supervision. ZV: validation, writing—review, and editing. All authors read and approved the final manuscript.

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CORRECTION
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Corrigendum: Impact of Zinc and/or Herbal Mixture on Ruminal Fermentation, Microbiota, and Histopathology in Lambs

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A Corrigendum on

Impact of Zinc and/or Herbal Mixture on Ruminal Fermentation, Microbiota, and Histopathology in Lambs

by Petrič, D., Mravčáková, D., Kucková, K., Kišidayová, S., Cieslak, A., Szumacher-Strabel, M., et al. (2021). Front. Vet. Sci. 8:630971. doi: 10.3389/fvets.2021.630971

In the original article, there was a mistake in **Table 2** and **Table 3** as published. **In the columns under m/z [M-H]- in all expressions separating numbers from decimals, there should be dots (.) not a slash (/).** The corrected **Tables 2** and **3** appear below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 2 | Concentrations of the main bioactive compounds in medicinal herbs (g/kg DM).

RT (min)	UV (nm)	*m/z*[M-H]$^-$	MS^2	MS^2 fragments	Formula	Compounds	Flavonoids	Phenolic acids
Fumaria officinalis								
7.00	250/326	295.046	179.0338		$C_{13}H_{12}O_8$	Caffeoylmalic acid		1.212
7.80	227/315	163.0395	119.0499		$C_9H_8O_3$	O-Coumaric acid		0.742
9.00	255/352	625.1398	301.0337		$C_{27}H_{30}O_{17}$	Quercetin-O-Hex-Hex	2.384	
9.40	255/352	595.1287	301.0339		$C_{26}H_{28}O_{16}$	Quercetin O-Pen-Hex	3.500	
9.90	252/351	609.1472	300.0279	285	$C_{27}H_{30}O_{16}$	Isoquercitrin O-Dhex		0.934
10.20	255/354	463.0882	301.0337		$C_{21}H_{20}O_{12}$	Quercetin O-Hex	1.706	
10.90	221/329	593.1520	285.0397		$C_{27}H_{30}O_{15}$	Kaempferol-3-O-rutinoside	0.464	
11.50	255/365	639.1561	315.0504		$C_{28}H_{32}O_{17}$	Isorhamnetin-O-Hex-Hex	0.558	
						Total contents:	12.211	3.961
Malva sylvestris								
7.00	523	757.1846	347.0761	329,261,509	$C_{32}H_{39}O_{21}$	Delphinidin 5-glucoside 3-lathyroside	1.644	
7.90	308	163.0381	119.0502		$C_9H_8O_3$	Coumarinic acid		0.468
10.00		609.1458	301.0330		$C_{27}H_{31}O_{16}$	Quercetin-3-O-rutinoside	0.395	
10.20	268/343	447.0928	285.0386		$C_{21}H_{20}O_{11}$	Kaempferol-O-Hex	0.494	
11.40	268/336	431.0978	269.0435		$C_{21}H_{20}O_{10}$	Apigenin-O-Hex	1.560	
						Total contents:	6.479	0.654
Artemisia absinthium								
4.10	215/325	353.0877	191.0567	179,161,135	$C_{16}H_{18}O_9$	Chlorogenic acid		3.416
11.00		515.1193	353.0867	191,179,135	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid		2.124
11.20		653.1719	345.0595	330,302	$C_{29}H_{34}O_{17}$	Spinacetin 3-rutinoside	0.241	
11.70		515.1192	353.0869	173,179,191,155	$C_{25}H_{24}O_{12}$	4.5-Dicaffeoylquinic acid		0.610
						Total contents:	0.349	6.482
Matricaria chamomilla								
4.30	215/300	353.0877	191.0567		$C_{16}H_{18}O_9$	3-O-Caffeoylquinic acid		1.777
9.00	235/290/319	355.1029	193.049	149	$C_{16}H_{20}O_9$	Methyl 4-O-beta-d-glucopyranosyl caffeate		3.202
9.70	255/354	463.0879	301.0337	151	$C_{21}H_{20}O_{12}$	Quercetin O-Hex	0.199	
10.30	257	447.0920	285.0386		$C_{21}H_{20}O_{11}$	Kaempferol O-Hex	1.363	
10.70	217/291/325	515.1189	353.0877	179,191	$C_{25}H_{24}O_{12}$	3.5-Dicaffeoylquinic acid		0.824
11.00	217/291/325	515.1197	353.0869	191,179	$C_{25}H_{24}O_{12}$	1.5-Dicaffeoylquinic acid		3.016
11.40	266/300	431.0976	269.0434		$C_{21}H_{20}O_{10}$	Apigenin O-Hex	0.150	
11.70	215/290/325	515.119	353.0868	173,179,191	$C_{25}H_{24}O_{12}$	4.5-Dicaffeoylquinic acid		0.851
14.40	218/268/339	473.1085	269.0427	406	$C_{23}H_{22}O_{11}$	Apigenin -O-(Hex-Ac)	0.210	
						Total contents:	2.442	12.084

TABLE 3 | Concentrations of the main alkaloids in *Fumaria officinalis* (g/kg DM).

RT (min)	UV (nm)	*m/z*[M-H]$^-$	MS^2	MS^2 fragments	Formula	Compounds	Alkaloids
7.70	272	354.1366	305.0811	279,233,323,336	$C_{20}H_{19}NO_5$	Parfumine	0.884
8.40	280	328.1572	265.0865	237,297,313,178	$C_{19}H_{21}NO_4$	Cularimine	0.102
8.50	288	370.1678	291.1029	263,352,337	$C_{21}H_{23}NO_5$	Fumaricine	0.102
8.90	285	326.1410	311.1172	277,294,251,178	$C_{19}H_{19}NO_4$	Cheilanthifoline	0.231
9.20	286	354.1360	275.0713	336,247	$C_{20}H_{19}NO_5$	Chelidonine	0.154
9.40	289	398.1621	338.1397	277,323,249	$C_{22}H_{23}NO_6$	Not determined	0.530
10.20	289	354.1362	275.0692	247,293,206	$C_{20}H_{19}NO_5$	Protopine	0.873
10.60	288	354.1727	206.1139	275,311,338,292	$C_{21}H_{23}NO_4$	Protopine type	0.367
10.80	271	352.1193	279.0647	309,321,263,251	$C_{20}H_{17}NO_5$	Fumariline	1.728
11.20	288	324.1230	249.0764	307,277,219,176	$C_{19}H_{17}NO_4$	Stylopine	0.785
						Total contents:	6.015

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Nutritional Aspects of Ecologically Relevant Phytochemicals in Ruminant Production

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This review provides an update of ecologically relevant phytochemicals for ruminant production, focusing on their contribution to advancing nutrition. Phytochemicals embody a broad spectrum of chemical components that influence resource competence and biological advantage in determining plant species' distribution and density in different ecosystems. These natural compounds also often act as plant defensive chemicals against predatorial microbes, insects, and herbivores. They may modulate or exacerbate microbial transactions in the gastrointestinal tract and physiological responses in ruminant microbiomes. To harness their production-enhancing characteristics, phytochemicals have been actively researched as feed additives to manipulate ruminal fermentation and establish other phytochemoprophylactic (prevent animal diseases) and phytochemotherapeutic (treat animal diseases) roles. However, phytochemical-host interactions, the exact mechanism of action, and their effects require more profound elucidation to provide definitive recommendations for ruminant production. The majority of phytochemicals of nutritional and pharmacological interest are typically classified as flavonoids (9%), terpenoids (55%), and alkaloids (36%). Within flavonoids, polyphenolics (e.g., hydrolyzable and condensed tannins) have many benefits to ruminants, including reducing methane (CH_4) emission, gastrointestinal nematode parasitism, and ruminal proteolysis. Within terpenoids, saponins and essential oils also mitigate CH_4 emission, but triterpenoid saponins have rich biochemical structures with many clinical benefits in humans. The anti-methanogenic property in ruminants is variable because of the simultaneous targeting of several physiological pathways. This may explain saponin-containing forages' relative safety for long-term use and describe associated molecular interactions on all ruminant metabolism phases. Alkaloids are N-containing compounds with vast pharmacological properties currently used to treat humans, but their phytochemical usage as feed additives in ruminants has yet to be exploited as they may act as ghost compounds alongside other phytochemicals of known importance. We discussed strategic recommendations for phytochemicals to support sustainable ruminant production, such as replacements for antibiotics and anthelmintics. Topics that merit further examination are discussed and include the role

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of fresh forages vis-à-vis processed feeds in confined ruminant operations. Applications and benefits of phytochemicals to humankind are yet to be fully understood or utilized. Scientific explorations have provided promising results, pending thorough vetting before primetime use, such that academic and commercial interests in the technology are fully adopted.

Keywords: feed additive, methods, nutrition, rumen modifiers, ruminants, antinutritive factor

INTRODUCTION

Ideal anaerobic fermentation in the rumen relies on a steady supply of substrate (i.e., quantity and frequency), preservation of a favorable condition for microbial growth (e.g., temperature, pH, substrate mixing), and constant removal of undesirable substances (e.g., bacterial toxins, hydrogen), so that the profile and amount of volatile fatty acids (VFA) produced and microbial protein leaving the rumen meets the ruminant's daily requirements for energy and protein without having deleterious impacts in the rumen health and functionality (e.g., rumenitis) (1, 2). Although the rumen can function adequately if these conditions are met, it may not be operating at its maximum anaerobic efficiency. Thus, some dietary tweaking might achieve maximum anaerobic efficiency or maintain a healthy operational rumen.

This is where feed additives, also known as rumen modifiers, come into play. If the feed additive is of plant origin, i.e., phytogenic, they are collectively referred to as phytochemicals. Some usually refer to them as plant secondary metabolites because they are not associated with essential roles in the plant, such as photosynthesis, respiration, and growth and development (3). However, the distinction between primary and secondary metabolites is obscure and relative to the plant's physiological needs. For instance, environmental conditions and ecological niches drive the synthesis of different phenolics that are entrenched in the plant's genome based on their evolutionary strategies, but the reasons for evolutionary demands, however, are unclear (4).

Phytochemicals of nutritional and pharmacological interest, such as those to prevent (phytochemoprophylaxis) or treat (phytochemotherapeutic) animal diseases, are typically classified as flavonoids (e.g., polyphenolics), terpenoids (e.g., terpenes), and alkaloids (3). Not all phytochemicals have known beneficial properties to ruminants, but those that do so are often grouped as polyphenolics (e.g., hydrolyzable—HT—and condensed—CT—tannins), terpenes (e.g., saponins), vitamins, and essential oils (EO). In part, the immense variability in phytochemical biological properties makes it very difficult to catalog them and study their effects on animals of economic relevance.

Flavonoids are polyphenolic compounds comprising fifteen carbons, with two aromatic rings (AC and B) connected by a three-carbon bridge, called flavan (**Figure 1**). About 5,000 flavonoids have been isolated (6), and the important ones are assigned to 12 subclasses, including anthocyanidins, aurone, chalcone, coumarin, dihydrochalcone, dihydroflavonol, flavan-3,4-diol, flavan-3-ol, flavanones, flavones, flavonols, and isoflavones (5, 7). Polyphenolics (e.g., tannins) comprise a significant subclass of flavonoids (**Figure 1**). Condensed tannins have been extensively used in ruminants because of their ability to reduce methane (CH_4) emissions (8, 9); shift protein digestion from the rumen to the small intestine (10, 11); improve the maternal environment and reproductive efficiency (i.e., ovulation, scanning, pregnancy, and fecundity rates) (12); support early embryonic survival (13); enhance embryo and fetal development, lambing rates, and lamb survival from birth to weaning (12); and trigger blood cell counts and the immune system response (14), among many other applications (1, 15, 16). Given the broad and sometimes incomplete understanding of CT's impact on the rumen's fermentation dynamics, interest has intensified in their ability to alter animal products' nutritional and organoleptic characteristics. The modulation of ruminal biohydrogenation with consequent alteration of the fatty acid composition of milk and meat is perceived as beneficial to humans because of the relative increase of omega-3, *trans*-11, and conjugated linoleic and linolenic fatty acids (17). Similarly, of particular interest is the ability of CT to reduce gastrointestinal parasite burdens (14, 18–20) given growing concerns of pharmaceutical antiparasitic resistance in grazing ruminants (21) due to their continuous, and sometimes unnecessary, treatment with ivermectin, a macrocyclic lactone.

Approximately 30,000 terpenoids compounds have been identified (6). Among these, saponins are classified into triterpene or steroidal glycosides, having many different bio-physicochemical properties. Most studies in ruminants have focused only on saponins' nutritional aspects to prevent digestive disorders when used as feed additives (22). Plant extract is the typical form adopted to achieve the desired phytochemical compound, and five saponin-rich plants have been consistently examined: *Camellia sinensis* L. (23, 24), *Quillaja saponaria* (25, 26), and *Sapindus rarak* DC.—lerak (27, 28) and *Sapindus saponaria* L.—soapberry (29, 30) with a triterpenoid structure; and *Yucca schidigera* (31, 32) with a steroidal nucleus.

In the last 15 years, some advancements in phytochemical research have been motivated by governmental regulations focusing on public health interests, especially those related to antimicrobial resistance (AMR) due to the broad and unbridled use of antibiotics in animal production as well as poorly controlled use for treating humans (16). Therefore, effective antibiotics replacements, including EO (33), such as allicin (garlic

Abbreviations: AMR, antimicrobial resistance; BW, body weight; CPP, ciliate protozoa population; CT, condensed tannins; DM, dry matter; DMI, dry matter intake; EO, essential oils; GIN, gastrointestinal nematode; HT, hydrolyzable tannin; TPS, triterpenoid saponins; and VFA, volatile fatty acids.

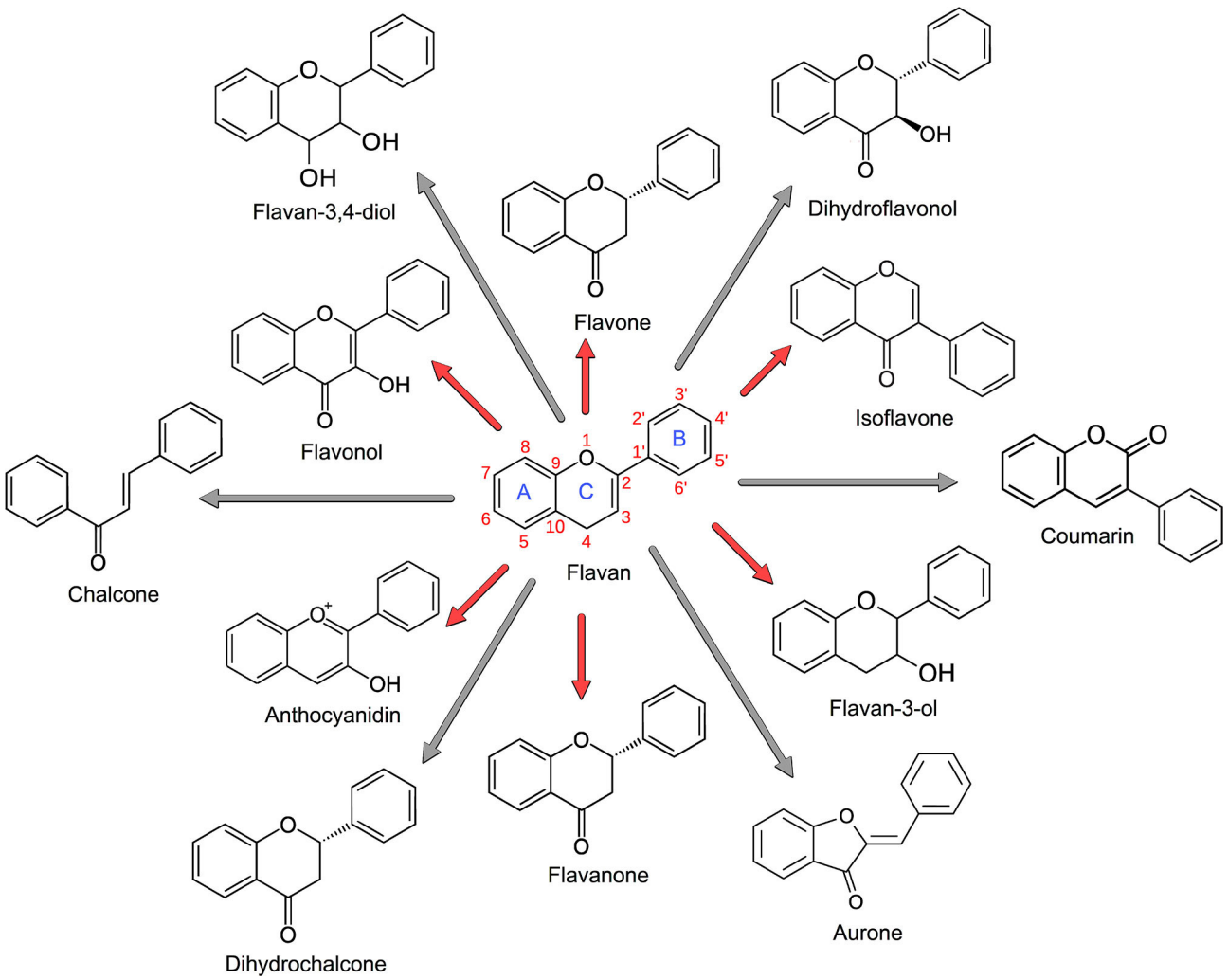

FIGURE 1 | Flavonoid nomenclature. The main subclasses of flavonoids are shown in red arrows, including anthocyanidins, flavan-3-ol, flavanones, flavones, flavonols, and isoflavones. The minor flavonoids are shown in gray arrows, including aurone, chalcone, coumarin, dihydrochalcone, dihydroflavonol, and flavan-3,4-diol. Isoflavones are characterized by having the B-ring attached at C_3 rather than the C_2 position, and coumarins follow the same structure of isoflavones, except for the double-bond oxygen that is located in C_3, not in C_4. Based on Hahlbrock (5) and Crozier et al. (3).

extract), carvacrol (oregano extract), cinnamaldehyde (cinnamon extract), and thymol (thyme extract), have been extensively investigated in broilers, pigs, and aquaculture (34, 35) due to their bacteriostatic and bactericidal properties. However, ruminant studies, including volatile terpenoids as EO constituents, have yielded mixed results (34, 36).

Alkaloids are nitrogen-based chemical compounds synthesized by plants for defensive purposes against predation by an offending organism, such as microorganisms, insects, herbivores, and sometimes, even other plants. Besides the deterrence of predation, there is growing evidence that alkaloids are also produced to harm the offending organism's growth and development through allelopathic action (37). Their toxic effect depends on their type and the amount consumed by the animal, but its primary purpose is to repel feeding via visual or olfactory signals (38). After ingestion and absorption, alkaloids can cause physiological and metabolic changes in the offending organism. Alkaloids can also be produced by animals, insects, and marine vertebrates, although plant extracts are the primary source that has been extensively studied (39). Most studies with alkaloids are related to their toxicological effect on animals rather than their phytochemical feed additive properties. Research on alkaloid pharmaceutical properties in humans was initiated in the 1980s (40). By the early 1990s, about 10,000 alkaloids were cataloged (41), including aconitine (anti-rheumatism), atropine (antispasmodic), caffeine (a stimulant), codeine (analgesic), ephedrine (decongestant), ergotamine (migraine), hydrastine (lower gastrointestinal disorders), and morphine (pain killer) to list a few (42). To date, more than 20,000 alkaloids have been isolated (6). However, few studies were conducted with domesticated animals to improve their production performance,

or alkaloids have acted as ghost phytochemicals with unknown biological importance.

This review aims to discuss important nutritional and methodological aspects of major phytochemicals relevant to ruminant production, including flavonoids (e.g., polyphenolics), which comprise 9% of phytochemicals (5,000 in 55,000), terpenoids (e.g., saponins, EO, and fat-soluble vitamins), which contain 55% of phytochemicals (30,000 in 55,000), and alkaloids, which comprise 36% of phytochemicals (20,000 in 55,000).

POLYPHENOLICS

Classification and Definitions

Polyphenolic plant secondary metabolites are ubiquitous throughout the plant kingdom. Tannins are a subclass in terrestrial plants broadly categorized into two major compounds: CT and HT. Hydrolyzable tannins are esters of gallic acid with a polyol core molecule, commonly glucose, and might be further categorized into ellagitannin, gallotannin, and galloglucose subclasses. Condensed tannins are polymers of flavan-3-ol (**Figure 1**) with subunits categorized as catechin, epicatechin, gallocatechin, or epigallocatechin. The diversity of the chemical structures of CT is vast. When considering the multiple subunit and bond types, a simple trimer could represent 1 of nearly 600 different isomers (43). Both CT and HT bind and precipitate protein via hydrogen bonding and hydrophobic interactions (44), a defining characteristic in tannin-ruminant animal interactions.

Nutritional Importance

The chemical properties of tannins contribute to nutritional and antinutritional effects on ruminant animals. The nutritional importance of tannins largely depends on their ability to bind to macromolecules and mineral nutrients. Condensed tannins and HT readily bind to dietary proteins in ruminants (45, 46) and interact with dietary lipids (47), polysaccharides (48), and metal ions (49). Tannins also alter microbiomes and inhibit microbial and enzymatic activity in the rumen (50, 51) and during the ensiling process (52).

Hydrolyzable tannins have often been regarded as potentially toxic, antinutritional plant secondary metabolites due to their tendency to be degraded in the rumen and absorbed by ruminants (53). More recently, however, potential benefits of HT on ruminant animal production systems have been reported (54–56). Unlike HT, CT have generally been considered as non-degradable by rumen microbes (57). However, possible ruminal degradation and total tract disappearance of CT have been reported. Robbins et al. (58) reported only 75% of CT consumed was recovered in feces of domestic sheep compared to >90% recovery in mule deer. More recently, and using much more sophisticated measuring systems, Kronberg et al. (59) determined that more than 90% of the CT consumed by sheep were degraded. Conversely, Desrues et al. (60) recovered all CT from sainfoin following total tract passage through cattle. Like many biological activities driven by CT, degradation in the ruminant digestive tract is likely dependent upon plant species, tannin type, and chemical structure. The variation in the survival of CT through the digestive tract point to the need for the strategical application of CT (i.e., nutritional vs. antiparasitic effects, rumen vs. post-rumen activity, CH_4 abatement vs. rumen protected N) rather than the commonly used "shotgun" approach. The fate of CT in ruminants and associated nutritional implications should continue to be a future focus of research on physiological and modeling research (15).

Ruminal Fermentation

Polyphenolic phytochemicals potentially offer numerous benefits to ruminant animal production. The most notable of those benefits is rumen microbiome modifiers (61) to alter gaseous emissions (62) and improve animal production (63). Much of the recent research on tannins has focused on the topic of rumen modification to mitigate greenhouse gas emissions and improve the N-use efficiency of ruminant livestock. The majority of this research has focused on the application of CT, but increased research interest in HT is becoming more evident.

Our knowledge and potential application of tannins in production scenarios are hindered due to a lack of understanding of how tannins interact with substrate and microbes in the rumen. Currently, CT are believed to reduce CH_4 production in the rumen by combining three possible mechanisms (43): (1) the formation of CT complexes with fermentable macromolecules and microbial enzymes, reducing the availability of substrates to microbial degradation, (2) the direct interaction between microbes and CT, resultant of CT binding to microbial lipopolysaccharide, and (3) CT subunits degrade in the rumen and become hydrogen sinks, reducing the hydrogen available to form CH_4 gas. The hydrogen-sink hypothesis has been demonstrated with catechin monomer subunits *in vitro* by Becker et al. (64). However, tannin scientists have yet to reveal the possibility that CT polymers undergo the necessary degradation in the rumen to become hydrogen sinks. Similar to CT, HT is thought to reduce enteric CH_4 by directly interacting with microbes or acting as a hydrogen sink (65). However, it is not believed that HT reduce CH_4 by decreasing substrate availability as a concomitant decrease in CH_4, and fiber digestion is typically not observed (66). However, our assumptions of how tannins behave in the rumen are continually evolving and require technological advancements and modeling techniques to understand the dynamic relationship better.

Recent research has focused on the application of respirometry methodologies to increase our understanding of the effects tannins have on CH_4 emissions *in vivo*. However, *in vivo* research has been inconsistent, with discrepancies among CT and HT studies being indicative of complex associations. For example, the use of quebracho CT extract has resulted in reports of reduced CH_4 emissions (62, 67) and no effect (68). Of these studies, beneficial effects were observed in those that fed a roughage diet and higher rates of CT (>1.5% DM). This may be, at least in part, an effect of CT rate or diet type but is likely a combination of the two; however, we lack conclusive data to understand this complex relationship. Similarly, HT in ruminant diets has also demonstrated varied results for CH_4 emissions. Recent work showed that gallic acid, an HT derivative, and tannic acid reduced CH_4 emissions in beef cattle (54, 69),

whereas HT from chestnut appears to have little or no effect (70). However, once again, we lack adequate data to conclude the reason for these differences. The discrepancies among CT and HT studies appear to point to a dynamic relationship among a variety of factors, including the chemical structure of the tannin, tannin inclusion rate, base diet (i.e., forage vs. concentrate), and animal species and stage of production (i.e., maintenance, growth, fattening, or lactation).

To garner a better understanding of the dynamic relationship among factors affecting tannin efficacy, *in vitro* gas production techniques have been used to screen tannin-rich forages for their potential to alter fermentation patterns and reduce CH_4 emissions by ruminants. This approach has proven to be a cost-effective and time-saving tool (71, 72). These techniques are especially useful when exploring domestication of wild types of perennial prairie legumes (73), investigating increased utilization of arboreal plant resources as forage (74), or when seeking the value of feeding invasive plant species to ruminants (75, 76). However, there is often some disparity between *in vitro* and *in vivo* CH_4 production (77). To better understand the real impact of tannins on ruminant nutrition, long-term and conclusive *in vivo* or *in situ* studies must be conducted to calibrate *in vitro* data. We must enable the application of *in vitro* methods to provide a rapid determination of tannin feasibility in various production systems.

Post-rumen Digestion

Ruminant animals are generally considered inefficient at converting ingested protein into an animal product due to a large portion being lost as NH_3 in the rumen. The efficiency of N use and retention by ruminants can be improved by either slowing the degradation rate of protein to enhance synchrony with carbohydrates or increasing rumen undegradable protein in the diet (78). Much of the interest in tannins revolve around the prospect of possible degradation and absorption of rumen undegradable protein following dissociation from tannin-protein complexes post-rumen. Condensed tannins readily decrease ruminal N digestibility (79), resulting in reduced urinary N excretion (62, 80) with a concomitant increase in fecal-N excretion and a possible reduction in excreta gas emissions (81). This shift in the site of N excretion might represent a decrease in N retention. However, some have reported increases in the efficiency of protein utilization expressed as weight gain per protein intake due to CT inclusion in the diet (82). Hydrolyzable tannins also bind and precipitate proteins, possibly increasing post-rumen availability of N, but they also offer the potential to slow the ruminal degradation of N and possibly promote the synchrony of N and carbohydrate degradation. Much like what is observed when feeding CT to ruminants, a shift in N excretion from urine to feces is observed when including HT in the diet (56). While increases in N utilization associated with feeding HT have not been reported, supplementation with gallic acid may decrease urinary N excretion without negatively impacting N digestibility (54).

The ability to shift the route of N excretion from the urine to the feces without sacrificing N digestibility is increasing in interest due to excreta's contribution to total livestock emissions. The feeding of CT has demonstrated the potential to decrease fecal gas emissions (81, 83) and reduce fecal urease activity (84). Similarly, nitrous oxide emissions from urine were reduced when gallic acid was fed (51). Once again, we lack adequate data to assume the mechanism(s) that alter emissions or that the observed alterations in excretion route and excreta emissions will improve overall emission status. However, based upon the positive results observed in the limited number of studies performed, research into the effect of tannins on excreta emissions warrants greater focus.

Gastrointestinal Nematodes

Gastrointestinal nematode (GIN) and other gastrointestinal parasite infections negatively impact ruminant nutrition. Both small and large ruminants are affected, but internal parasites are especially detrimental to small ruminants, including sheep and goats. Legume CT, particularly those from sericea lespedeza (*Lespedeza juncea* var. sericea), demonstrate anthelmintic activity against GIN parasites in small ruminants (85). Sericea lespedeza (*Lespedeza cuneata*) (86) and quebracho (*Schinopsis* sp.) (87) CT also inhibits *Eimeria* spp. in goats, which are responsible for coccidiosis. Condensed tannins may also be efficacious as an anthelmintic against common cattle parasites *Cooperia oncophora* and *Ostertagia* spp. (88).

Increased use of the larval exsheathment assay has led to the screening of novel forage CT for anthelmintic activity (89). *In vitro* screening for the potential anthelmintic activity of tannin-rich forages is not limited to CT. Concentrations of 25 mg HT/ml effectively kill *Haemonchus contortus in vitro* (55). Gallic acid reportedly demonstrates egg hatch inhibition against GIN that commonly infect cattle (90).

An important question that cannot be answered using *in vitro* techniques is what are the negative nutritional and toxic implications, if any, of feeding HT to ruminants for GIN control? Therefore, *in vivo* research must follow reports of positive impacts of CT but especially HT to confirm anthelmintic activity without detriment due to phytochemical toxicity. Some hypothesize that parasitized ruminants will intentionally select forages with anthelmintic properties (i.e., tannins). Some evidence of ruminant self-medication by selecting for tannin-rich forages when parasitized by GIN has been reported (91). More often than not, however, the self-medication hypothesis is not confirmed (92, 93).

Why and When Do Tannins Work?

The question of when and why tannins positively impact ruminant nutrition is a difficult one to answer. Tannin bioactivity, especially that of CT, is often plant-specific. The mechanisms of action for tannin biological activities, such as ruminal CH_4 mitigation, reducing rumen proteolysis, or inhibiting GIN, are mostly unknown. The mechanism for one biological activity likely differs from that of another.

There is evidence that structurally recalcitrant tannins are most effective in modifying fermentation and reduce CH_4. For example, CT from *Acacia angustissima* var. *hirta* are highly effective at reducing enteric CH_4 production (73). The undegradable 5-deoxy flavan-3-ol structure of the *Acacia* CT

likely contributes to its ability to mitigate CH_4 formation during fermentation (94). Additionally, tannins' antioxidant activity is positively correlated ($r > 0.90$) to ruminal CH_4 emission (94), suggesting that antioxidant activity at least contributes to the mechanism of action involved in CT-CH_4 mitigation.

The ability of tannins to bind and precipitate protein, and potentially create rumen undegradable protein, may depend on factors associated with chemical structure and conformation, the pH in which the tannin-protein interaction occurs, and the herbivore's ability to bind tannins with salivary proteins during mastication and rumination. The structural diversity of tannins adds to the difficulty of determining the impact of specific structural characteristics on tannin-protein interactions. Structural attributes of CT, including the mean degree of polymerization, stereochemistry, and prodelphinidin-to-procyanidin ratio, sometimes do not explain protein-tannin interactions (95). However, some reports suggest that large prodelphinidin-based CT demonstrate greater protein precipitating capacity than large procyanidin-based CT (96). Recently, the impact of the increased mean degree of polymerization and inter-flavan bond type on protein precipitation capacity has been confirmed (97). Similar to factors affecting protein precipitation by CT, larger polymers of HT demonstrate greater protein precipitating capacity than monomeric forms (98).

The pH is an important factor affecting the protein precipitating capacity of tannins; the closer to the isoelectric point of the protein, the greater the protein precipitation capacity (99, 100). Much of what we know about the role of pH in CT-protein interactions supports the hypothesis that protein-tannin complexes dissociate in acidic environments (such as in the abomasum of ruminants), leading to protein degradation and subsequent amino acid absorption in the small intestine. As the pH of the environment where tannin-protein complexes occur becomes more acidic relative to the isoelectric point of the protein, the protein precipitating capacity of tannins decreases (100). Accordingly, when the environment is less than pH 5, tannin-protein complexation may be minimal (101).

The neutralizing effect of proline-rich protein in saliva has long-been hypothesized (102). Many browsing herbivores that readily consume tannins do not produce saliva that contains proline (103). Despite a lack of proline, some browsing ruminants (i.e., goats) can bind tannins with salivary proteins (104), suggesting that proline is not a requisite for all salivary protein-tannin interactions.

An explanation for when and why tannins are useful anthelmintics continues to be elusive. Much of the literature suggests the efficacy of both CT and HT against GIN is dose-dependent (55, 89), such that greater concentrations of tannin result in more significant anthelmintic effects. Tannin concentrations vary within species based on plant maturity, which is another factor to consider. In some species, mature plants produce lower tannin concentrations than immature plants (76, 105), whereas others may increase or remain unchanged with maturity (105). However, it is crucial to fully understand the CT concentration at different seasonal growth stages in a given plant species (106) to maximize their ontogenic phytochemical characteristics on sustainable ruminant production systems (8).

Why Do Tannins Not Work?

Dietary tannins do not always affect the nutritional status of the ruminant animal. There are many possible reasons for this. If the forage or feed resource is too low in tannin concentration, little, if any nutritional impact will be observed. The tannin's chemical structure produced by a given plant can determine whether or not the phytochemical is effective at eliciting a desired animal response. Modes of action of tannins also differ for different activities such that the type and structure of tannin used to elicit one nutritional response may not be useful for that of another. Another challenge occurs when feeding highly bioactive tannin-rich forages. The animal may reject tannin-rich forage due to reduced palatability due to salivary protein binding and astringency.

Future Perspectives

There is still much to learn about how CT and HT affect ruminant animal nutrition. Much of what we understand about tannin impacts on ruminant nutrition is the result of *in vitro* studies. While *in vitro* assays are excellent screening tools, more *in vivo* confirmation of research findings is needed to move tannin science from use-inspired basic research to application. A significant challenge to this progress is the lack of domesticated (cultivated) plants rich in bioactive tannins. As a result, the availability of plant material suited for many ruminant producing regions is limited. Even when the seed is commercially available, it is often cost-prohibitive due to the limited supply and labor required to collect undomesticated species.

Future research should emphasize the strategical application of tannins rather than the current "shotgun" approach from a nutritional perspective. Much of the previous and recent research has emphasized directly inhibiting enteric CH_4 production and increasing rumen undegradable protein. However, there is potential to utilize some tannins' degradation to reduce CH_4 via hydrogen-sink and increase N-use efficiency by improving nutrient synchrony. There are opportunities to exploit tannins' antioxidant properties, particularly immunomodulatory effects, thermal stress, and human-health products. Tannins' influence on excreta emissions requires attention, but ultimately we need to understand better how excreta from animals consuming tannins alters soil fertility, soil microbiota, and plant growth.

Despite deficiencies in current knowledge about nutritional implications in ruminant animals, polyphenolic phytochemicals (i.e., tannins) have great potential as a tool in ruminant production systems. Further investment in plant breeding and domestication efforts, as well as research efforts to further elucidate how tannins impact ruminant nutrition and system processes, will be necessary to realize the full potential of these important phytochemicals.

TERPENES

Biosynthesis and Functionality

The bitter-taste, emulsifying, foaming, non-ionic, non-volatile, membranolytic, surfactant, and structurally diverse saponins (glycosides) are low molecular weight (1,000–1,500 Da) secondary natural compounds in food and non-food plants (107–109), marine plants (110) and animal lineages, including invertebrate sea cucumber species (111, 112). Chemically, glycoside saponin biosynthesis begins with the catalyzation of acetyl co-enzyme A to isopentenyl pyrophosphate units generated by the multistep mevalonate 3-hydroxy-3-methylglutaryl-CoA reductase (113), a common route to the synthesis of cholesterol and some steroids (114).

Saponins comprise the hydrophobic aglycone (sapogenin) structure that is linked to polar functional groups and attached via a 3-C chain structure to an individual or multiple hydrophilic sugars (i.e., arabinose, galactose, glucose, glucuronic acid, methylpentose, rhamnose, or xylose) (115, 116) and moieties (i.e., glycones) (117, 118). Aglycones are subject to gene encode enzyme-mediated (i.e., cytochrome P450-dependent glycosyltransferases, monooxygenases, and others) (119) change (i.e., acylation, hydroxylation, glycosylation, oxidation, and substitution) (119, 120) to form a varied group of compounds (121).

Saponins are chemically categorized into two groups: triterpene or steroidal. Following the isoprenoid pathway, the aglycone splits into pentacyclic triterpenoid saponins (**TPS**) with a 30-C aglycone core by cyclization of 2,3-oxidosqualene (113, 117, 122), yielding the first group of saponins. The second group is related to the biosynthetic pathway of tetracyclic steroidal metabolites to a 27-C aglycone backbone (114, 117, 120) with a 5-ring furostane or a 6-ring spirostane skeleton (123) involving oxygenations and glycosylations (117).

Although in the presence of other phytochemistry (124), saponin mixture in a single plant species occurs (120, 121, 125), such as cucurbitane, cycloartane, dammarane, holostane, hopane, lanostane, lupane, oleanane, tirucallane, taraxastane, tirucallane, and ursane TPS types (107, 126) have been identified in more than 500 plant species (114). Within a hundred family-group plants, the Anacardiaceae, Araliaceae, Combretaceae, Compositae Campunalaceae, Caryophyllaceae, Leguminosae, Polygalacea, Sapindaceae, Theaceae, and Verbenaceae families, their genera and species attract more attention (114, 127–131).

In angiosperm monocotyledons and angiosperm dicotyledons plants, the variation, composition, concentration, distribution, and differential bio-activity of TPS are influenced by plant growth, agronomic and genotype-environmental interactions (132–134). Moreover, TPS-plant storage, physical milling, TPS separation, and the bio-accessibility of metabolites in the form of concentrated extracts, derivatives, or food additives to facilitate human-animal utilization may modify aglycones' structure and their bio-physiological, nutraceutical, and pharmaceutical activities (121, 124, 135).

Although paths for those roles are not well-understood and despite differences in chemical structures, different activities exist for TPS, including adjuvant (136), antibacterial (137, 138), antidiabetic (139), antifungal (140–142), anti-inflammatory (123, 125), antioxidative (109, 143), antiprotozoal (144–146), antiproliferative (147), antiviral (148, 149), cardiotonic and cardioprotective (122), and cytotoxic (127, 128, 150) effects have been reported. Additionally, TPS have also exhibited other functional properties, such as food-additive in flavorings (26), gastroprotective (151, 152), hemolytic (153), hepatic (139, 149), immunologic (123, 154), insecticide (155, 156), anti-obesity therapeutic potential (111, 116, 157, 158), neuroprotective (159), vermicide (160), and emulsifier and stabilizer of the nanosuspensions (161, 162).

Nutritional Importance

Central to TPS's bio-physicochemical network of interactions, the nutritional significance of TPS for ruminants stems largely from their digestive and methanogenic significance (163). Consequently, using *Medicago sativa* L. (alfalfa) and *C. sinensis* L. (tea plant) as examples, this review will be limited to considering certain aspects of the bio-metabolic and rumen microbial shifts in sheep and cattle derived from TPS supplementation, which are not entirely consistent and understood. Compared to non-supplemented diets, **Table 1** has a comparative overview of digestive function reaction to alfalfa-TPS (26.9–601.3 mg/g extract) intraruminal or feed-mixed supplemented [10.6–800 mg TPS/kg body weight (**BW**)] in different breeds and BW (42–60 kg) of sheep between 14 and 90 days.

Based on the use of 17.8–35.9 mg TPS/g extract, an intraruminal increasing TPS-dose in wethers fed roughage diets resulted in a less disturbed digestive system than the digestive responses of intraruminal supplemented wethers fed concentrate diets (164). However, using 27.8 mg TPS/g extract, compared to the lowest intraruminal dose of 200 mg/kg BW in Suffolk wethers fed grass-hay, 800 mg/kg BW administered intraruminally increased rumen pressure, particulate matter outflow, and VFA concentration by 25, 25, and 10%, respectively (115). This effect was further associated with a reduction in organic matter (11%) and neutral detergent fiber (10%) total tract digestibilities, ciliate protozoa populations (**CPP**; 80%), and daily CH_4 production (8%) (115).

There is limited experimental data on the use of TPS on animal production under mid to long-term management. However, Liu et al. (108) demonstrated complementary opportunities for both physio-metabolism and production evaluation. These authors indicated that a high-TPS concentration extract shifted from 0.04 to 0.08 TPS-to-dry matter intake (**DMI**) ratio in concentrate plus roughage diets used by Hu male-lambs during 90 days, yielded a 12, 44, 2, 2, and 7% increase in dry matter (**DM**), neutral and acid detergent fibers, ether extract, and crude protein digestibilities, respectively. Nevertheless, when the TPS supplementation increased from 24 to 94.3 mg/kg BW, the effects on DM, neutral and acid detergent fibers, ether extract, and crude protein digestibility decreased by 12, 44, 2, 2, and 7%, respectively. These effects were also associated with an 8% reduction in daily BW gains.

These studies illustrate how sheep responses can be influenced by motivated, focused action. However, the long-range vision

TABLE 1 | Effects of triterpenoid saponin (TPS) supplementation on several ruminal and total gastrointestinal tract parameters[1].

Plant species[2]	TPS-animal interaction					Digestive parameters							
	Extract	TPSC	Animal	mg/kg BW	TPS:DMI	RM	RPC	PMO	OMD	NDFD	TVFA	CPP	DMP
Medicago sativa L.[a]	Root	27.8	Sheep[‡T]	200	1	1.74	+22	0	−1	−2	+6	−42	+6
				400	2	1.67	+21	+4	−7	−8	+3	−81	+5
				800	3	1.67	+53	+25	−12	−12	+17	−88	−2
					TPS:DM	RTR	TMRT	DMD	OMD	HEMD	CELD	TVFA	CPP
M. sativa[b]	Plant	26.9	Sheep[†]	10.6[*]	2	−6	0	−1	−1	−2	−6	+1	−37
				21.4[⁂]	4	−3	−2	0	0	−1	+4	−6	−47
				10.6	2	−24	+10	+6	+1	+35	+32	−19	−33
				21.4	4	−23	+7	+9	+2	+28	+40	−28	−55
					TPS:DMI			DMD	NDFD	ADFD	EED	CPD	
M. sativa[c]	Leaf-root	601.3	Sheep	12.0	0.04			−1	−15	+15	0	+2	
				24.0	0.08			+11	+22	+17	+2	+9	
				47.1	0.16			+4	+1	−24	−2	+3	
				94.3	0.32			−3	−8	−15	−5	−4	

[1]*Ratios of TPS to dry matter (DM) (TPS:DM) or TPS to DM intake (DMI) (TPS:DMI); rumen motility (RM, n/min)[†]; rumen pressure change (RPC, mm Hg)[†]; ruminal turnover rate (RTR, %/h); total mean retention time (TMRT, h); particulate matter outflow (PMO, g/d); total-tract crude protein (CP), DM, ether extract (EE), organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), hemicellulose (HEC), and cellulose (CEL) digestibilities (g/100 g); total volatile fatty acids (TVFA, mmol/L); ciliate protozoal populations ([‡]CPP × 10^5/ml); and daily methane production (DMP). The notations + refers to an increase and – refers to a decrease in percentage values relative to non-TPS supplemented diets data in each experiment. Triterpenoid saponin concentration (TPSC) is presented in mg/g of M. sativa plant extract; and mg/kg DM of M. sativa root extract, and M. sativa leaf-root commercial extract product.* [2]*a = Klita et al. (115) in which intraruminal TPS extract supplementation was conducted for 14 days. [†]Measured on day 11. [ᵀ]Methane measurements (24 h) on day 12 based on indirect calorimetry and respiratory hoods from 4 Suffolk wethers. b = Lu and Jorgensen (164) in which *roughage and [⁂]concentrate diets fed to wethers subject to intraruminal daily supplementation of TPS during 14 days. c = Liu et al. (108) in which TPS-supplemented concentrate plus roughage diet were fed twice daily to 10 Hu male-lamb groups (n = 5) during 90 days.*

to shape or reshape TPS's use and ensure its relevance to small ruminant needs a particular combination of knowledge and perspectives. It should equate the sheep feed industry interest with clinical science in the context of a deepening sense of animal practice responsibilities to concomitantly address societal needs and ecosystem environmental challenges.

Overall, we can only speculate that the TPS-extract source within the same plant species, the extraction method, compound composition, concentration and dose, way and time of supplementation, diet type, and sheep genetics refer to the range of variation in the summarized alfalfa-TPS supplementation response in **Table 1**. Unless such information is forthcoming, there is a risk of limiting factors to benefit from the TPS functional activities described above with sheep if they are susceptible to specific doses in farming grazing conditions.

Table 2 illustrates how cattle and sheep respond to TPS supplementation. It illustrates the impact of TPS doses from tea seeds and alfalfa extract sources on fermentative, microbial, and blood parameters of Brahman (*Bos indicus*) and crossbred *B. indicus* cattle (234–364 kg) and sheep (41.7–42.5 kg). The approach is justifiable because, in the current and post-COVID challenges, it is unlikely that individual research could undertake simultaneous cattle-sheep TPS supplementation assessments. However, it would be possible for cooperative research across the livestock industry to justify the expense involving additional knowledge gains.

As with beef cattle, sheep can cope with increasing doses of tea seed-TPS. A difference is the range of TPS doses tested between large and small ruminants. Another critical difference is the greater emphasis on cattle measurements after TPS withdrawal than on sheep. This has resulted in the interaction among supplementation digestive and fermentative parameters. The summarized data indicate that Ramos-Morales et al. (169) pointed out that TPS does not always reduce CPP. However, this information may not be surprising because saponin functional diversity and biological pathways do not always positively correlate (170). Early on, Dourmashkin et al. (171) provided evidence that saponins at 0.05% concentration modify eukaryotic cell membrane permeability by producing a pore-forming characteristic expected to inhibit both CPP (115) and CH_4 emissions (172).

Published trials using tea seed-TPS indicated that their anti-methanogenic effect *in vitro* (173) in small ruminants (167, 168, 174) is considered to be a selective saponin-sterol association (175, 176) on protozoa surface (170). Nevertheless, CPP may increase when plant-TPS (145, 177) and low cell-wall carbohydrate diets are fed (178).

Dourmashkin et al. (171) found that saponin-treated cell membrane growth is associated with concentrations above 0.09%. Sidhu and Oakenfull (179) also demonstrated that, when orally fed, saponins are not absorbed into the bloodstream but might modulate mitosis (180, 181) by molecule transport, cell membrane fluidity, and cell proliferation *in vitro* (182) and *in vivo* (183).

Contrary to the transient antiprotozoal effect of TPS (184), a linear increase of protozoal numbers is triggered by increasing tea seed-TPS doses in crossbred Brahman cattle, while a defaunation effect was observed at 13 days post-TPS treatment

TABLE 2 | The effects of supplementing triterpenoid saponin (TPS) from *Camellia sinensis* L. or *Medicago sativa* L. on digestive and blood profiles of †Belmont Red Composite [Africander (African Sanga) × Brahman (*Bos indicus*) × Hereford-Shorthorn (3/4 *B. taurus*)] and ⸸Brahman steers, and ‡Dorper crossbred × thin-tailed Han ewes, ⸸Hu rams, ᵀHuzhou lambs and *Hu male-lambs[a].

Parameters[b, c]	***C. sinensis*[b, c]**					
	Cattle†			**Sheep‡**	**Sheep⸸**	**Sheepᵀ**
TPS mg/kg BW	31.5	44.3	Post-TPS	28.6	83.7	112
TPS:DMI	0.14	0.22	Post-TPS	0.13	0.18	0.30
TVFA	+3	−2	−2	+16	−3	+13
CPP	+99	+190	−5	−16	−42	−41
DMP	+6	+3	−16	+2	−11	−27

Animal species	***C. sinensis* serum biochemistry**											
	TPS mg/kg BW	**TPS:DMI**	**CL**	**K**	**Na:K**	**I**	**GLU**	**CHO**	**UM**	**GGT**	**ALP**	**AST**
Cattle⸸	22.7	0.11	−1	−12	+12	−23	−15	+5	−29	+5	+5	−5
	44.2	0.21	+1	−11	+12	−21	−15	0	−17	+10	+10	−7
	64.9	0.30	+3	−12	+13	−22	+3	+5	−20	+17	+17	0
	***M. sativa* plasma profile**											
			GH	**IGF-1**	**T3**	**T4**	**GLU**	**CHO**	**UN**	**TRG**	**ALT**	**AST**
Sheep*	12.0	0.04	+38	+32	+77	+42	+20	+2	+5	−2	+46	+31
	24.0	0.08	−9	−5	+22	−9	+16	−7	−30	−35	+49	+27
	47.1	0.16	+8	+2	+11	−18	+20	−9	−44	−68	+81	−27
	94.3	0.32	+5	−8	+40	+4	+21	−3	−35	+3	+98	−28

[a] *Positive and negative percentage data refer to non-TPS supplemented diets in each experiment.*

[b] *Ciliate protozoal populations (†CPP Log × 10^8/ml rumen fluid, ᵀCPP a % of total bacterial 16 S rDNA, ⸸CCP × 10^5/ml, ‡CCP × 10^7/ml), daily methane (CH_4) production (DMP), dry matter intake (DMI), body weight (BW), total volatile fatty acids (TVFA, mmol/L). Serum electrolytes and minerals [mmol/L; chloride (CL), potassium (K), sodium to potassium ratio (Na:K), iron (I, μml/L)]. Metabolites [mml/L; cholesterol (CHO), glucose (GLU)]. Renal function [mmol/L; urea nitrogen (UN)]. Enzimes [IU/L; alkaline phosphatase (ALP), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT)]. Plasma hormones [ng/mL; growth hormone (GH), insulin-like growth factor-1 (IGF-1); mmol/L; tri-iodothyronine (T3), and thyroxine (T4)]. Metabolites [mg/dL; urea nitrogen (UN), glucose (GLU); mmol/L; triglyceride (TRG), alanine transaminase (ALT), aspartate aminotransferase (AST)].*

[c] *† = Ramírez-Restrepo et al. (165) in which eight rumen-cannulated steers were progressively supplemented with dissolved tea seed saponin (TSS; 580 mg TPS per g of TSS) mixed in the morning diet during 3 and 4 days. Post-TPS values were recorded 13 days after TPS withdrawal. Individual CH_4 emissions were measured (48 h) in open-circuit respiratory chambers, recording levels of supplementation of 27.0 and 43.5 mg TPS/kg BW, which are equivalent to ratios of 0.13 and 0.23 TTS:DMI, respectively. ⸸ = Ramírez-Restrepo et al. (166) in which after 13.8 mg TPS/kg BW (0.08 TPS:DMI) supplementation during 6 initial days, a gradual increase of intraruminal (four cannulated steers) dissolved TSS supplementation before the morning feeding and mixed in the morning feed (2 non-cannulated steers) was performed during 7, 14, and 16 days, respectively. ᵀ = Mao et al. (167) in which 32 lambs fed in two equal parts daily. Open-circuit respiratory chamber measurements (48 h) and microbial populations from four lambs after 60 days trial. ⸸ = Zhou et al. (168) in which 12 rumen-fistulated Hu rams fed once a day. Three rumen-fistulated and re-faunated Hu rams supplemented with 1.8 g of TPS for 3 weeks in the basal diet. Open-circuit respiratory chamber measurements (24 h). * = Liu et al. (108) in which TPS-supplemented concentrate and roughage diets fed AM and PM to 10 Hu male-lamb groups (n = 5) during 3 months; physiological values in the 60–90-day period. ‡ = Liu et al. (108) in which 18 primiparous and six rumen-cannulated Dorper × thin-tailed Han crossbred ewes were used and fed supplemented for nutrient digestibility and CH_4 emissions in open-circuit respiratory systems (Experiment 1, 29 days), and fermentation and microbial ecology examination (Experiment 2, 42 days), respectively.*

as shown in **Table 2** (165). There, TPS modified the structure of the methanogen community at the subgenus by increasing the numbers of methanogens and decreasing their abundance in the RO and SGMT clades, respectively (185). In parallel, TPS supplementation reduced numbers of protozoal genus *Entodinium* spp. and increased *Euplodinium* and *Polyplastron* genera. The withdraw of TPS supplementation was associated with lower proportions of *Isotricha* and the greater presence of *Metadinium* and *Eudiplodinium* genus (185).

This suggests that, in tropical cattle, TPS may have a high selectivity index for protozoa, without an adaptation of those ciliates and other microbial communities to short-term feeding of TPS. Moreover, it is essential to note that tea seed-TPS as a feed additive appears to exert a differential protozoal and anti-methanogenic effect across Dorper × thin-tailed Han crossbred ewes, Hu rams, and Huzhou lambs (**Table 2**). With these facts in mind, readers are directed to Hu et al. (173), Guo et al. (172), Mao et al. (167), Zhou et al. (168), Wang et al. (186), and Liu et al. (187) for the detailed complementary impact of TPS on rumen ecology and extend of nutrient digestion. Together, these findings mirror the belief that further research is required to understand better multifaceted TPS supplementation effects associated with the breed, sex, and animal category sound interactions.

Future Perspectives

Although in our research no comparison of patterns of CH_4 emissions was performed between a single and two equal daily portions of TPS supplementation, there is little doubt that the circadian rhythm of CH_4 emissions from steers after the morning non-supplemented and TPS-supplemented diets (165) is consistent with that observed in twice-daily TPS-supplemented

sheep (167, 168, 188) and cattle fed Rhodes grass (*Chloris gayana* Kunth) *ad libitum* (189). Conversely, the current review provides evidence that forage diets fed to ruminants could modulate the animal response to TPS-sources inclusion in tropical agriculture (177, 190–192). However, this reason may be further explained by capturing TPS supplementation advantages in seasonal nutrition, fermentability, and methanogenic indices of forages (71, 72). A sustainable ruminant industry should consider three questions. How long does the TPS-protozoal selective effect in the rumen ecosystem of tropical cattle last? Is this physio-metabolic response opening the possibility that tea seed-TPS may reduce cattle CH_4 emissions in the long-term rather than as an immediate abatement? Should we investigate the effects of very low TPS concentration additives and/or far lower TPS:DM ratios on ruminants to achieve target microbial community profiles without significant associated meta-physiological disturbances?

Few ruminant studies beyond methanogenesis have focused on complementary clinical responses to TPS supplementation (**Tables 1**, **2**) to understand or confirm pharmacological discoveries, phytochemical screening, safety, and efficiency of therapies, and *in vitro* findings. In particular, safety and tolerability studies have demonstrated that Brahman (166) and Belmont Red Composite [Africander (African Sanga) × Brahman × Hereford-Shorthorn (3/4 *B. taurus*)] (165) steers tolerate on average 32.2 ± 16.61 and 27.3 ± 13.53 mg/kg BW of TPS supplementation during 23 and 20 days, respectively. This for each breed is ∼6.4 and 4.5 vs. 5.5 and 3.8-fold the non-toxicological effect levels in mice (i.e., subcutaneous injection) (117) and dogs (i.e., intramuscular route) (193), respectively.

However, as low TPS doses in Brahman (13.8 ± 0.64 mg/kg BW) and Belmont Red Composite (9.2 ± 0.35 mg/kg BW) steers are 1.9 and 1.2%; respectively, of the canines long-term daily administration (i.e., 26 weeks), this variation might facilitate further efforts to clarify biological constraints and a vision of improved farming practices. In parallel, TPS effects on animal behavior and health indicated that the administration at 0.42 ± 0.013% of the DMI to Brahman steers remarkable reduced DMI, and developed primary tympany and enteritis.

Although that high dose was not tested on Belmont Red Composite steers, a similar clinical pattern of symptoms but a lower magnitude were experienced when TPS doses achieved between 0.10 and 0.14 ± 0.003% on the DMI. This was consistent with other studies (194, 195) that reported that some TPS might disrupt endothelial permeability, infiltration of cellular systems, and active nutrient transport, and nutrient uptake in the gut. This likely involves a sequential cascade involving cytokines, chemokines, reactive oxygen species expressions, and several intracellular signaling pathways, to name a few (196). However, those cattle dose-dependent effects contrast Klita's et al. (115) reports that sheep have a lethargic feeding behavior and lack of rumination associated with intra-ruminal TPS:DMI ratios of 4 and 8%.

The interaction between TPS and the functional capacity of organs and body systems can produce relatively complicated outcomes. **Table 2** underpins blood test differentiation between TPS-plant sources and animal species. That strategy should, in turn, allow greater understanding of significant differences in blood biochemistry and biological drivers between non-cannulated and cannulated cattle after TPS supplementation (166). Based on the evidence provided here, it appears that such physiological associations could be the vehicle to spread knowledge and refine and collect prolonged assessments to ensure practical use of TPS additives.

Collectively, in response to the natural structure of TPS and their related sapogenins (126, 169, 170, 184), possible reasons for the observed differences within bovids are the pharmacodynamic and pharmacokinetic profile expressions of the host physiological system (197, 198). This is likely characterized in healthy animals by differential genetic and metabolic binding, inter-individual variability, cellular and molecular self-regulatory feedback mechanisms, induction and inhibition of pathways, pharmaco-genomics, and pharmaco-metabolomics (199).

However, supported by the heterogeneity of systemic reactions shown in **Tables 1**, **2**, it is suggested that a broad medical approach in future studies is critical to understanding TPS supplementation throughout the interrelationships within and between ruminant species, breeds, and crossbred animals. Medicine will benefit from increased knowledge of more significant or down-regulation expression of signal transducers, transcription factors, membrane proteins, ion channels, and mitochondrial enzymes in cell lines (200, 201). Such observations further indicate the relevance of complementary microbiota analysis to understand the impact on ruminal ecology, methanogenesis, and animal physiological functioning following clinical-relevant TSS-supplementation and at withdrawal endpoints.

In summary, although over the last years, review research advances in TPS have been evident (27, 162, 202–208), the disparities in physicochemical characteristics of close and non-closely intermediate related compounds in TPS-containing plants (209–211) from one to another material depends on the vast structural diversity of TPS molecules (131, 212). Therefore, feasible investigations should focus on TPS physio-metabolic interactions after ingestion to elucidate complex interactions with the diet's nutritive value and substantial variation in gastrointestinal microflora and animal metabolisms. This is reasonably straightforward in intermolecular forces, genetic-molecular animal predispositions, cellular signaling frameworks, intra-cellular-matric chemoreceptors, metabolic fluxes, multi-enzyme cascade, and morphological changes. The approach across the catalog of TPS-plants, their phytochemical compounds, and interactions will promote secondary compound-physiological-based ruminant models (15), human and animal health, regulatory environments, ecosystems management, and eco-efficient ruminant production.

VITAMINS AND ANTIOXIDANTS

Types of Vitamins

Various vitamins and related minerals, many of which play critical roles as antioxidants important for growth and health, are sometimes deficient in ruminant diets. Ruminant requirements change with species, class, age, weight, health, and growth performance (213), but much of the research into these requirements are outdated and not representative of current production systems. Vitamin and related mineral deficiencies most often affect animals fed in confinement and only rarely occur in those allowed to graze or browse pastures and rangeland containing abundant, diverse plant species except when soils are severely deficient, as is sometimes the case with Se (214). When deficiencies occur, they are often a result of incorrect ration formulations or antagonistic effects (e.g., K and P, S, and Cu). However, they can be corrected by supplementation, feed changes, or allowing animals access to diverse pastures containing dicotyledenous species, such as legumes. Historically, cattle confined feeding operations have supplemented ruminants at or above published requirements as a preventative measure (215).

In grazing or browsing ruminants, most vitamins and minerals necessary in cellular antioxidant activity can be ingested from fresh plant material. In turn, these are transferred to ruminant products; dairy products especially can accumulate these compounds, often quantified as antioxidant protection degree (214) or total antioxidant capacity (216). Unsaturated fatty acids, phenols, and volatile compounds are likewise transferred from forages to dairy products and play important roles in taste and odor as well as eventual consumer health (217). These are incredibly rich in grazing systems, at times ten times greater than in stall-fed ruminant diets (218). Therefore, vitamin and mineral supplementation often becomes the best management option only in confined feeding operations or monoculture grazing systems.

Importance

The α-tocopherol and related compounds (vitamin E) and closely associated selenium (Se) are common feed-related deficiencies in confined ruminants not fed fresh green forages (219). Both are important in antioxidation processes that mitigate stress. Vitamin E, in conjunction with Se, plays a crucial role in cellular antioxidation. When deficient, physiological and immunological functions can be impaired, as can growth performance in confinement (220).

Retinol (vitamin A) is fat-soluble and plays an important role in ruminant eyesight, bone development, epithelial cell function, reproduction, as well as general immune functions (221). In ruminants, retinol enhances antioxidation that protects against cellular free-radicals (222). Carotenes are retinol precursors, and, under pasture or rangeland conditions, over 10 carotenoids have been documented in forages that can meet ruminant requirements (221). Their presence in milk produces distinctive butter and cheese colors that consumers identify with grazing-based dairy. However, feeding trials in confined feeding systems where fresh, green forage was lacking indicate that retinol supplementation to sheep (223) and calves (224) increases its presence in animal tissue, indicating that deficiencies may occur. There is also evidence that Vitamin A can interfere with Vitamin E retention in ruminant blood plasma, liver, and fat tissue.

Ascorbic acid (vitamin C) inhibits cortisol release, is a robust cellular antioxidant, and plays a vital role in ruminant products' fatty acid profile, especially dairy (225). Its supplementation to confined ewes, for example, increases the antioxidant concentration in milk (226). It also affects lamb, but not kid, meat quality parameters when administered before transport and slaughter (227). Diet can be a strong determinant of herbivore blood and milk ascorbic acid concentrations (228, 229), and its injection in confined cattle can reduce mortality rates (230).

Folic acid and vitamin B_{12} supplemented to confined multiparous (older) dairy cows can reduce dystocia by 50% and speed up first breeding postpartum by 3.8 days (231). It has no effect on primiparous dairy cows or any other health or reproductive factor for either class of animals. This indicates that, in confined feeding conditions, these can be essential supplements in multiparous ruminants where vitamin B can become depleted over time. No similar positive effect of folic acid and vitamin B_{12} supplement in pastured ruminants has been observed.

The Ruminant Animal's Perspective

Stress on ruminants affects animal health by increasing cellular oxidation. Stresses include abiotic factors, such as climate (mainly temperature extremes) or management, including transport or handling (219). Biotic stresses include interaction with other animals, reproduction, lactation, and feed quantity and quality deficiencies, as well as numerous other potential interactions with the living environment. Oxidative stress occurs when reactive oxygen species or free radicals surpasses the detoxification capacity of antioxidants. Activation of inflammatory-immune response and decreased overall immune function can result. There is evidence indicating that oxidative stress during weaning and transport plays a crucial role in the occurrence of bovine respiratory disease (232, 233) and affects feed efficiency (234) in newly received feedlot cattle. Ingesting antioxidants, such as vitamin E and related Se, can help reverse these adverse effects. When these are limited in the diet of confined ruminants consuming a limited diversity of fresh forages, supplementation can mitigate the adverse effects of stress on growth and product quality (226, 235, 236).

The Ruminal Microorganisms' Perspective

Ruminal microbes can synthesize as well as degrade vitamins and other antioxidative dietary compounds. Diet affects this dynamic. High energy concentrate diets, for example, result in an 80% vitamin A loss in the rumen compared to only 20% breakdown in high-forage diets (237). As a result, slow-release rumen boli containing vitamins and minerals have proven effective for enhancing confined ewe reproductive functions (238). However, it is unclear if vitamins played any role and their effectiveness declines after the initial weeks. The effectiveness of slow-release Cu, Se, or Co has proven especially useful in pastures where soils and consequently forages are low in any one of these minerals.

However, because forages typically supply vitamins above rumen microorganism requirements, their supplementation has not been widely studied in grazing or browsing ruminants. In a feedlot where fresh forages are rarely an ingredient, however, this picture changes drastically.

The Consumers' Perspective

Volatile compounds ingested by grazing and browsing lactating ruminants change milk and dairy product fatty acid profile and antioxidant properties (239–241). Not only can this extend product shelf life, but it can also be important for health benefits to consumers as well as unique flavors in milk, butter, and cheese that arise from consuming certain forages that vary by region and season (214). These are driven by forage composition, particularly dicotyledonous plant species (242). When animals are fed in confinement, supplementation can compensate for vitamin deficiencies in the animal, which is then reflected in the product (235). In North American milk production, where strong flavors are not a consumer preference, forages containing these compounds may not always be desirable.

Sources of Vitamins

The role of Vitamin E and other antioxidants in ruminant nutrition and health has been well-documented. Without them, animal health suffers, and production yield and quality decline. What is not always recognized is that their supplementation is largely irrelevant to pasture or rangeland-fed animals that ingest these naturally from fresh, green forages. These antioxidants readily appear in products originating from these free-ranging ruminants (214). Grazing and browsing ruminants, especially in ecosystems with diverse plant species, rarely benefit from dietary supplements. The same is not the case for confined feeding operations or monoculture grazing systems.

Confined animal feeding operations for feeding ruminants high energy diets invariably enhance animal production and health when they include synthetic vitamins and other antioxidant-enhancing supplements in the feed. This will come from fresh green forages or, in their absence, as synthetic supplements. These are generally injected to increase efficiency and bypass rumen degradation, but slow-release ruminal boli may also play a role in systems that do not lend themselves to repeated injections (238).

Very little is known about the antioxidant efficacy of feeding conserved (e.g., hays and silages) vs. freshly harvested (greenchop) forages to confined ruminants. Feeding trials comparing cut-and-carry or greenchop systems to conserved forages should also examine the role of forage species, functional groups (e.g., legumes), plant maturity, environment (e.g., soil nutrients or moisture), browse vs. grazing (especially for goats), and species diversity. Additional trials should examine the benefits of allowing animals to graze, browse, or even pen-feed selectivity (self-medication) for forages that lend themselves to greater antioxidant activity in the ruminant, animal products, and humans who consume products containing high or low concentrations of ruminant-originating antioxidants. Additional research should compare the efficacy of plant vs. synthetic vitamin sources in ruminant diets.

Should vitamins be systematically quantified in ruminant feedlot diet components? Quantifying vitamins important in ruminant cellular antioxidant functions in confined animal feed may not be as useful as measuring key minerals, mostly because the former is broken down by rumen microorganisms fed high concentrate and high energy feeds, making these unavailable for absorption in the remainder of the gastrointestinal tract. Supplementing vitamins up to minimum recommended levels has already been proven beneficial to ruminants in confinement, under heavy reproduction pressure, or under management-induced stresses, such as handling or transport.

Future Perspectives

Additional research topics needing attention include the effectiveness of slow-release rumen boli for vitamins in feedlot systems. Timing (reproduction, weaning, season, maturity), rumen microorganism breakdown leading to inefficiencies, and duration of release all merit attention. The efficacy of slow-release supplements for confined feeding vis-à-vis fresh forages (classes, species, maturity, diversity) also merits focus, especially regarding animal and human consumer health benefits.

The key question is, should we invest resources in this phytochemical? For pasture-based systems that include diverse forage species, including legumes and other forbs, any investment is unlikely to produce any measurable benefit except in cases where soils are deficient in key minerals, such as Se, important for antioxidant health. More research is needed in the case of confined feeding operations, especially long-duration systems, such as confined dairies. Examples include comparing the economic and health returns of year-round fresh, diverse forage systems where mild climates allow cultivation during any season.

ALKALOIDS

Classification and Definitions

Alkaloids represent the largest class of secondary plant compounds in North-American perennial plants and occur in many rangeland grasses and weeds (243), where they mostly have gained attention as a potential toxin for ruminants and other pasture livestock in case of overfeeding of alkaloid-containing plants. Alkaloids were initially classified as cyclic compounds containing N in a negative oxidation state, derived from an amino acid. However, some pseudo-alkaloids are not derived from amino acids and alkaloid-like compounds (amines) that do not contain N within any ring-structure. Given the confusing nomenclature of alkaloids, pseudo-alkaloids, and amines, it seems more convenient to classify them based on their biogenetic origins, where four groups were created: (1) alkaloids derived from ornithine, arginine, lysine, histidine, phenylalanine, tyrosine, tryptophan, anthranilic acid, and nicotinic acid; (2) purine alkaloids (e.g., xanthine caffeine); (3) aminated terpenes (e.g., diterpene aconitine, triterpene solanine); and (4) polyketide alkaloids (e.g., coniine, coccinellines) (39). Alkaloids may be produced by plants and fungi infesting certain pastureland plants, such as the endophytic fungus *N. coenophialum* in tall fescue that contains the alkaloids peramine, ergot, and loline (244).

Nutritional Importance

Overall, forage plants that include significant concentrations of alkaloids are considered toxic as many adverse effects in livestock exist, including acute and chronic symptoms, such as damage to the central nervous system, liver damage, muscle cramps, and death (245). The toxicological effects associated with alkaloids, specifically the broadly present class of pyrrolizidines, has been in discussion since the 1960s, specifically in context with animal production (246). Specifically, breeding efforts to remove tannins from forage for ruminants to optimize meat production may have possibly reduced tannins and alkaloids' interactions, thus increasing the toxicity of the latter (247). Many plants with high alkaloids in the leaf are not palatable to herbivores due to bitterness (248). It has been observed that wild animals (e.g., deer, rabbits) tend to limit the consumption of alkaloid containing plants but also to be highly tolerant. This resistance to chronic alkaloid intoxication has been, in part, ascribed to intestinal microbiome containing strains that can degrade alkaloids (249).

Initial efforts to remove alkaloids from the food chain of livestock production did not consider the crucial role of alkaloids across several ecological networks (245). Ergot alkaloids (e.g., ergovaline, ergonovine, ergine) are commonly found in tall fescue (*Festuca arundinacea*—now *Schedonorus arundinaceus* Schreb.; https://plants.sc.egov.usda.gov), but an endophytic fungus—*Neotyphodium coenophialum*—produces them. Through a mutualistic symbiotic relationship, it enables the tall fescue to thrive during drought and cold weather and resist insect predation, nematode infestation, and some diseases (244), but it can be devastating to the ruminant animal (250, 251). In a previous study, a genetically modified non-producing-ergot *N. coenophialum* has been incorporated into tall fescue to still yield the plant's agronomic benefits without causing toxicity to the grazing animal (244). Similarly, perennial ryegrass (*Lolium perenne* L.), another widely used cool-season pasture grass, is infected with *N. lolli*—an endophyte fungus that produces the biologically active ergot, peramine, and lolitrem alkaloids, which cause ryegrass staggers in livestock (244). In contrast, reed canarygrass (*Phalaris arundinacea* L.) produces the alkaloid gramine in leaf sheaths and stems, reducing ruminant's forage intake, thus limiting growth and development (244).

Simultaneously, various therapeutic activities have been ascribed to alkaloids, including antioxidant, cancer-preventive, antidiabetic, anti-inflammatory, and vasodilatory activities (252–254), but it has not been well-investigated how livestock could benefit from these beneficial activities from alkaloids. Many plant extracts that have been investigated for the beneficial actions of contained polyphenols and terpenoids may also contain alkaloids contributing to their biological activities, for example, giant milkweed (255) or herbal mixtures containing polyphenols, terpenoids, and alkaloids (256).

Additionally, the microbiome of ruminants, including bacteria, archaea, protozoa, and fungi, in part, metabolizes alkaloids to non-toxic metabolites (257); however, causal relationships have not been well-investigated. For example, Koester et al. (258) showed that cows with high vs. low tolerance to fescue toxicosis have vastly different microbiota compositions, specifically fungal phylotypes Neocallimastigaceae, potent fiber-degrading fungi, were consistently more abundant in the tolerant cattle. Additionally, it has not been well-investigated, which microbial enzymes are required to perform alkaloid metabolism (259).

Future Perspectives

Overall, alkaloids' beneficial role to ruminants and their synergistic contributions to ecological networks in forage-animal management has not been well-investigated. The contribution of alkaloids in complex plant extracts beneficial to ruminant nutrition also remains to be explored.

ESSENTIAL OILS

Classification and Definitions

Unlike the previous phytochemicals that maintain a reasonably specific chemical makeup, EO are mixtures of compounds comprised of previously discussed phytochemicals and other intrinsic chemicals. Indeed, the nomenclature "essential oils" is a misnomer because EO is neither essential in the sense that animals have a daily requirement nor are oils because they contain glycerol (2). The term EO was likely derived from quinta essentia (i.e., quintessence) attributed to Bombastus Paracelsus von Hohenheim$^{1493-1543}$, who used the term for any extraction of pharmacological drugs via steam distillation (260). Essential oils are classically defined as complex, multi-component mixtures of various volatile and non-volatile compounds, including acids, acetones, alcohols, aldehydes, esters, phenolics, and terpenes (261). The primary constituents of EO are low molecular weight terpenes/terpenoids and aromatic compounds, with monoterpenes representing 90% of EO (262). Essential oils are commonly extracted from materials found throughout the plant, including bark, leaves, flowers, roots, seeds, and stems. The biological properties of an EO are determined by its chemical profile that can vary depending upon the extraction process, plant material, plant maturity, and growing environment (262). In many cases, much of the pharmaceutical properties exhibited by EO can be attributed to the phytochemicals that comprise an EO (e.g., terpenes, terpenoids, phenolics, polyphenolics) (261).

Essential oils can exhibit antimicrobial, antiseptic, antiparasitic, antioxidant, anti-inflammatory, and immuno-modulating activities. In general, EO display hydrophobic or lipophilic attributes that result in a high affinity for bacterial cell membranes, generating ion leakage that can ultimately result in ATP depletion and cell lysis (263, 264). Since ancient times, EO have been exploited by humans for their pharmaceutical properties (263), with EO currently being used regularly in agriculture, cosmetic, food, homeopathic, pharmaceutical, and therapeutic industries (262). Essential oils are cited as improving animal health and nutritional status by stimulating the circulatory, digestive, and immune systems, as well as reducing pathogenic bacteria and parasites (261, 265).

Nutritional Importance

The nutritional effects of EO are primarily attributed to their antimicrobial properties that are comprised of multiple interaction mechanisms. Gram-positive bacteria are considered

more susceptible to EO than gram-negative bacteria due to both hydrophobic and lipophilic interactions affecting cell membrane stability (266). However, small molecular weight components, via hydrophobic interactions, may be able to penetrate and affect gram-negative bacteria (267). The application of EO in ruminant nutrition has focused on ruminal modulation to shift the microbial consortium toward one that improves nutrient use efficiency (268). Significant emphases have primarily remained focused on N-metabolism, CH_4 abatement, and the VFA profile (36, 264). Essential oils' complex and varied composition may provide the potential to alleviate tolerance and resistance developments associated with medically important antimicrobials and synthetic compounds.

Ruminal Fermentation

The basis for employing EO in ruminant diets is to modify the microbial population so that efficient fermentation pathways are used, and the animal's nutrient use efficiency is increased. The primary means of accomplishing this is by altering the VFA profile (lower acetate-to-propionate ratio) and a reduction in fermentative waste products (e.g., CH_4 and NH_3). The mode of actions provided by EO suggests they may be able to modify ruminal fermentation similar to ionophores by decreasing the prevalence of Gram-positive bacteria, including hyper-ammonia producing bacteria and those that readily produce formate or H_2 (269).

In vivo research has demonstrated that EO reduce the acetate-to-propionate ratio to a level comparable to ionophores when ruminants are fed high-quality diets (e.g., dairy and feedlot) (270–274). However, this result is inconsistent, and it is not easy to discern if the decreased acetate-to-propionate ratio results from reduced acetate, increased propionate, or both, as all scenarios have been observed. An increase in butyrate has also been indicated in some studies (270, 275) and is cited as an indication that EO and ionophores have differing modes of action (264, 270). As well, ruminal branched-chain volatile fatty acids have been reduced (270) and increased (276, 277) *in vivo*, indicating an alteration in the cellulolytic microbes or those that synthesize branched-chain volatile fatty acids from branched-chain amino acids. Both branched-chain volatile fatty acids and branched-chain amino acids are essential for the normal fermentative functions of cellulolytic microbes in the rumen (1). Overall, the addition of EO often imparts no change to the total VFA concentration (277, 278). However, increased (271, 279) and reduced (280, 281) total VFA concentrations have been reported, but the reduction in total VFA concentration is typically not to the extent observed with ionophores (2, 22, 282).

The effect of EO on digestibility is a significant point of contingency, but it has not been a focal point for much of the *in vivo* work in beef cattle. Of those that have examined digestibility, there was no effect on DM digestibility or neutral detergent fiber digestibility (271, 272, 283). The result is similar in dairy cattle, with only marginal effects on digestibility (274, 281, 284, 285). As with digestibility, EO's inclusion does not appear to affect significantly intake, at least not at the supplementation levels commonly used *in vivo*.

The provision of EO *in vivo* has not demonstrated a repeatable effect on ruminal CH_4 without suppressing digestibility. Supplementing diets with EO has decreased CH_4 in dairy cattle (286–289), but did not change of increased CH_4 production in beef cattle (277, 278). Although CH_4 production has not been measured, when feeding EO, protozoa and methanogen numbers decline *in vivo* with a corresponding reduction in the acetate-to-propionate ratio (270, 272). A reduction in CH_4 without inhibiting digestion has typically been observed when EO are provided at ∼500 mg/kg DM, but as little as 41 mg/kg DM has imparted an effect. The beneficial effects are thought to be due to selective inhibition of protozoa and methanogens; however, the negative or ineffectual results are likely the result of EO demonstrating indiscriminate binding or lack of adequate biological activity.

Much research has investigated the potential application of EO to reduce proteolysis and deamination in the rumen. However, the consensus indicates that EO have little-to-no effect on the ruminal breakdown of protein and amino acids in beef or dairy cattle. The vast majority of research indicates no difference in ruminal NH_3 when EO are included in the diet (275, 278–280). Similarly, numerous studies have failed to indicate a difference in blood or milk urea N from animals provided EO (274, 290, 291). The lack of effect is thought to result from EO being supplemented at too low of a rate to alter N metabolism (264). However, reduced ruminal digestibility had no effect on ruminal NH_3 or blood urea nitrogen levels in beef heifers supplemented with EO (292, 293). This could indicate that some species of hyper-ammonia-producing bacteria are less sensitive to EO (294).

Post-rumen Digestion

Essential oils increase the flow of non-microbial N to the small intestine, as well as stimulate digestive enzymes and alter microbial populations in the lower tract. However, minimal investigation of rumen outflow and post-rumen digestion has been performed, particularly *in vivo*. In beef heifers, a linear increase in the flow of non-microbial N to the duodenum has been observed with an increasing rate of eugenol or cinnamaldehyde (292, 293). However, post-ruminal N digestibility does not improve when feeding EO (292, 293, 295, 296). The inclusion of EO yields equal or lesser ruminal N digestibility with no difference in intestinal digestibility. This results in total-tract N digestibility not different or lower than the control. A similar trend is present for starch and neutral detergent fiber digestibility, ruminally and post-ruminally. However, increased total-tract acid detergent fiber digestibility has been observed and attributed to a stimulatory effect of EO on digestive enzymes (283, 291, 297). In ruminants, no research has directly investigated EO as a stimulus for gastric or intestinal enzymes. However, this is not implausible as EO have demonstrated the ability to reduce pathogenic fecal bacteria (298) and diarrhea in calves (299), as well as reduce fecal DM and viscosity in dairy cattle (274).

Methodological Aspects

Essential oils have primarily been investigated using *in vitro* methods, batch or continuous culture, particularly when screening multiple compounds and rates. In many instances, batch incubations have not adequately represented the dynamic rumen environment, whereas continuous culture has provided fermentation and outflow data comparable to *in vivo* results. Over the past decade, *in vivo* methods have been regularly implemented, but efforts have mainly focused on dairy and feedlot sectors, with little to no investigation into grazing beef cattle. Much of the *in vivo* research focused on fermentation parameters and digestive functions has utilized low animal numbers (4–16) in Latin square or switchback designs. Some larger pen-fed studies have emphasized performance and carcass characteristics with digestive attributes being investigated with a small number of cannulated animals. A shortcoming of multiple fermentation studies is that the use of low animal numbers has not greatly progressed our knowledge of the inter-animal variation associated with EO provision. For *in vivo* investigations, the length of EO or treatment provision varies greatly. Research focusing on digestion and fermentation commonly utilized 14- to 31-day feeding periods, whereas the larger performance trials typically ranged from 80 to 205 days on feed. There is an apparent deficiency in fermentation and microbiota data for animals fed EO for more than 30 days, limiting our knowledge of digestive or microbial alterations with prolonged feeding.

Research Data

The successful application of EO depends on numerous factors, but the overall effect of EO is unclear due to a lack of consistency among measured variables. Even so, EO have regularly increased intake and improved the VFA profile and feed conversion in feeder cattle, as well as reduced CH_4 and increased milk yield and feed conversion in dairy cattle. The reason for the different effects between a feeder and dairy cattle is likely, at least in part, a result of differences in diet composition, particularly the level and type of roughage. However, there is little information to assist in making comparisons to high-roughage diets. In a meta-analysis of the essential oil blend, Agolin Ruminant®, in dairy cattle, Belanche et al. (300) determined that supplementation of the EO blend increased milk yield and reduced CH_4, and an adaptation period of at least 4 weeks was required for consistent results. Unfortunately, most digestive studies have utilized periods spanning 3–4 weeks, perhaps not allowing enough time for consistent outcomes to be realized. In a meta-analysis investigating the effects of EO in sheep diets, it was determined that EO increased neutral detergent fiber digestibility and propionate concentration and reduced protozoa populations and acetate concentrations (301). However, in contrast to dairy cattle, EO efficacy in sheep appeared to be highest within the first 30 days and then began diminishing. Regardless of species, the methodologies commonly used to study fermentation have not progressed our knowledge of EO efficacy with prolonged feeding.

Future Perspectives

There appears to be potential for EO to improve animal efficiency and performance, but the variation among studies makes it difficult to parse out the effect of EO vs. random variation. If EO are to be commercially used in ruminant production, emphasis should be placed on using methods that improve the consistency of results (i.e., increased replication, extended feeding periods, recovery methods, chromatographic methods to determine purity). Research using forage diets merit increased attention, as improved fermentation profiles would greatly benefit this sector. Apart from nutrition, there seem to be EO opportunities for internal and external parasite control. Multiple *in vitro* studies have reported acaricidal activity of EO and have successfully used them to control cattle ticks (302–304), with evidence indicating EO as a potential method of controlling flies and lice (305–307). This is a vital area of research due to the rapid increase in parasite resistance to synthetic compounds, providing a large opportunity to investigate feed-through and topically administered EO in ruminant species. Another area that merits attention is the effect of EO on thermal stress. There is scientific and anecdotal evidence indicating that EO's provision may reduce the stress associated with hyperthermia (308–310), but the underlying mechanisms and efficacy in a production scenario are unknown. Although EO's nutritional effects may not be consistent, there is potential for EO to improve other health parameters that directly and indirectly affect the nutritional status of ruminants.

Should researchers invest resources in EO? Based on the current literature, adequate data point to the benefits of EO to ruminant production. Additional efforts should invest in the long-term and diversity of these compounds. However, research projects must be performed in a manner that better capture the effect of EO and promotes consistency among trials, rather than focusing on the least publishable unit.

CONCLUSION

Many scientists embarked on alternative replacements to antibiotics in animal operations in the last 15 years after widespread concern over AMR due to antibiotics' perceived broad use in animal production. Phytochemicals became the preferred research pursuit, even though these compounds have been studied and applied in many fields long before AMR became publicized. Phytochemicals embody a broad spectrum of chemical components produced by plants and some fungi to act as chemicals against predatorial microbes, insects, and herbivores. Therefore, the idea of using them to manipulate ruminal fermentation and to establish other phytochemoprophylactic (prevent animal diseases) and phytochemotherapeutic (treat animal diseases) activities gained sympathizers.

Flavonoids comprise only 9% of known phytochemical compounds, but most research has been dedicated to this group, especially CT. However, because of inconclusive or contradictory findings, more targeted research is needed to confirm and validate published findings before definitive recommendations of phytochemicals usage in ruminant nutrition are drawn, such as what, when, and how much to use. Although some discoveries are

encouraging, disagreements and lack of repeatability exist among studies, particularly for CT and saponins.

Alkaloids may also have a potential untapped benefit in ruminant nutrition. Although humans have long used alkaloids for their pharmacological properties, their phytochemical usage as feed additives in ruminants has not been sufficiently scrutinized. In part, given the intricacies in measuring and classifying alkaloids chemically, they may act as ghost compounds alongside other phytochemicals of known importance as plants produce many phytochemicals concurrently.

Likewise, terpenes, vitamins, essential oils, and other natural plant antioxidants play a large role in rumen ecology and function. These are most prevalent but least studied in fresh forages, especially in rangelands. The difficulty of isolating their individual effects in forage-based systems make them especially challenging to describe. This, however, does not detract from the critical roles they plan in ruminant ecosystems. The importance and individual effects are more easily identified in feedlot situations where concentrates and preserved forages contain fewer compounds, with consequent adverse effects on rumen microbiome health and ruminant nutrition. More research in these compounds in concentrated animal feeding operations is therefore merited.

The phytochemicals' role in sustainable ruminant production is undeniable, but much uncertainty remains. Scientists have yet to answer the sustainability issues before relying exclusively on phytochemicals as a sensational remedy for AMR, especially in complete rations lacking fresh forages and precluding ruminant feed selection. Phytochemical feed additives may have a place in sustainable production scenarios only if more convincing results of their efficacy and effectiveness in replacing antibiotics are dependably identified. The old saying "do not put all your eggs in one basket" still applies to phytochemical research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Effect of Dried Distillers Grains With Solubles and Red Osier Dogwood Extract on Fermentation Pattern and Microbial Profiles of a High-Grain Diet in an Artificial Rumen System

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The objective of this study was to evaluate the effect of dried distillers grains with solubles (DDGS) and red-osier dogwood (ROD) extract on *in vitro* fermentation characteristics, nutrient disappearance, and microbial profiles using the rumen simulation technique. The experiment was a completely randomized design with a 2 × 2 factorial arrangement of treatments and four replicates per treatment. A basal diet [10% barley silage, 87% dry-rolled barley grain, and 3% vitamin and mineral supplement, dry matter (DM) basis] and a DDGS diet (as per basal diet with 25% of wheat DDGS replacing an equal portion of barley grain) were supplemented with ROD extract at 0 and 1% (DM basis), respectively. The experimental period was 17 d, consisting 10 days of adaptation and 7 days of data and sample collection. The substitution of wheat DDGS for barley grain did not affect gas production; disappearances of DM, organic matter, and crude protein; total volatile fatty acid (VFA) production; and microbial protein production. However, replacing barley grain with wheat DDGS increased ($P = 0.01$) fermenter pH and molar proportion of branched-chain VFA, switched ($P = 0.06$) the fermentation pattern to higher acetate production due to increased ($P = 0.01$) disappearance of neutral detergent fiber (NDF), and decreased ($P = 0.08$) methane (CH_4) production. In the basal barley diet, the ROD extract increased the acetate to propionate (A:P) ratio ($P = 0.08$) and reduced the disappearance of starch ($P = 0.06$) with no effect on any other variables. No effects of ROD in the DDGS diet were observed. The number of operational taxonomic unit (OTUs) and the Shannon diversity index of the microbial community had little variation among treatments. Taxonomic analysis revealed no effect of adding the ROD extract on the relative abundance of bacteria at the phylum level with either the basal diet or DDGS diet, while at the genus level, the microbial community was affected by the addition of both DDGS and the ROD extract. *Prevotella* and *Fibrobacter* were the most abundant

genera in the basal diet; however, *Treponema* became the most abundant genus with the addition of the ROD extract. These results indicated that the substitution of wheat DDGS for barley grain may mitigate enteric CH_4 emissions. The trend of reduced starch fermentability and increased NDF disappearance with the addition of ROD extract suggests a reduced risk of rumen acidosis and an improvement in the utilization of fiber for cattle-fed high-grain diet.

Keywords: red osier dogwood extract, DDGS, high grain diet, RUSITEC, fermentation pattern

INTRODUCTION

Red osier dogwood (ROD; *Cornus sericea*) is a native shrub plant that is present across North America and is abundant in low wetlands, pasturelands, and areas where crops and forages do not grow well (1). This plant is rich in bioactive compounds, with total phenolic concentrations of up to 22% of dry matter (DM), including anthocyanins, gallic acid, ellagic acid, quercetin, kaempferol, and cyanin (1). Gallic and ellagic acids have been shown to possess anti-inflammatory (2) and antioxidant (3) benefits (4). In recent years, ROD and its potential as a feed ingredient for livestock have been evaluated in *in vitro* and *in vivo* studies utilizing beef cattle and swine. Wei et al. (5) investigated the effect of substituting ROD for silage at 3% and 6–12% in a high-grain diet on *in vitro* rumen fermentation with varying media pH (5.8 vs. 6.5). They suggested that the inclusion of ROD at a media pH of 5.8 had greater effects on starch digestion than on fiber digestion. This is of particular interest as decreased ruminal starch digestion would alleviate rumen acidosis in animals that are fed high-grain diets, where the rumen pH is consistently below 5.8 and fibrolytic bacteria are compromised, potentially impacting the performance, as well as the health and welfare, of animals. Wei et al. (6) also reported that feeding ROD to beef heifers that are fed high-grain diets decreased the degradability of ruminal protein (i.e., increased the rumen bypass protein) and increased blood antioxidant capacity and the immune response. These authors suggested that the increase in rumen bypass protein was due to the protein-binding capacity of ROD phenols, thereby improving protein efficiency in cattle. However, these previous studies used raw ROD material, and to our knowledge, ROD extract and its effect on rumen fermentation have not been evaluated.

Dried distillers grains with solubles (DDGS) is the major by-product of ethanol production when corn or wheat grain is used as a substrate for ethanol production. The inclusion of DDGS in feedlot diets is a common practice, given its high protein and high digestible fiber concentrations, in addition to its competitive cost as a feed ingredient. However, diets with high inclusion levels of DDGS, especially wheat DDGS, which have a higher crude protein (CP) content than corn DDGS, often exceed CP requirements for cattle fed with barley grain-based feedlot finishing diets. Feeding CP in excess of cattle requirements is not nutritionally and environmentally desirable as it increases ruminal absorption of ammonia nitrogen (NH_3-N) and, thus, increases nitrogen (N) excretion in a volatile form (7). We hypothesize that supplementation of ROD extract in high-grain diets containing DDGS may alter the ruminal fermentation pattern in a desirable manner and increase ruminal bypass protein by reducing the NH_3-N concentration due to decreased proteolytic activity. Thus, the objective of this study was to investigate the effect of wheat DDGS, the supplementation of ROD extract, and their interaction on fermentation pattern, nutrient disappearance, and rumen microbial profile in an artificial rumen stimulation technique (RUSITEC).

MATERIALS AND METHODS

Experimental Design, Diets, and ROD Extract

The experiment was a completely randomized design with a 2 × 2 factorial arrangement of four treatments with four replicates, which included two total mixed ration diets; a basal diet (87% dry-rolled barley grain, 10% barley silage, and 3% vitamin and mineral supplement; DM basis) and a wheat DDGS diet (62% barley grain, 25% wheat DDGS, 10% barley silage, and 3% supplement; DM basis), each supplemented with 0% and 1% ROD extract (DM basis), respectively. The ROD extract was supplied by Red Dog Enterprise Ltd. (Winnipeg, MB, Canada) and was prepared using hydrothermal treatment with an extraction temperature of 98°C. The ROD extract contained approximately 6.7% moisture, 21.8% total phenolic content (expressed as gallic acid equivalents), 1.67% gallic acid, 0.70% ellagic acid, 2.75% rutin, 0.32% quercetin malonyl glucoside, and 0.01% quercetin [DM basis; (8)]. The inclusion level of the ROD extract was based on a previous *in vitro* study carried out at our laboratory (9). The total mixed ration diets were ground through a 4-mm sieve (Arthur Thomas Co., Philadelphia, PA, USA) and weighed (10 g DM) into nylon bags (10 × 20 cm; pore size of 50 μm, Ankom Technology Corp., Macedon, NY, USA). The ROD extract was then added to nylon bags at the desired level and was manually mixed. The chemical composition of the experimental diets is reported in **Table 1**.

Experimental Apparatus

Two RUSITEC apparatus were used with each unit equipped with eight 920-ml fermenters as described by Czerkawski and Breckenridge (10). Each fermenter had an inlet for the infusion of the buffer and an outport for the collection of effluent. Fermenters were immersed in a water bath maintained at 39°C. The four treatments were randomly assigned to duplicate fermenters within each RUSITEC apparatus. The experimental

TABLE 1 | Ingredient and chemical compositions of the experimental diets (% of DM unless otherwise stated).

Item	Basal	DDGS
Ingredient		
Barley silage[a]	10	10
Barley grain, ground[b]	87	62
Wheat DDGS[c]	0	25
Supplement[d]	3	3
Chemical composition		
DM, %	85.4	85.0
OM	94.3	92.3
CP	13.7	19.6
NDF	21.8	23.9
ADF	8.4	9.1
Starch	52.6	38.5
Ether extract	2.1	2.9

DDGS, dried distillers grains with solubles; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber.

[a]Composition (DM basis): 38.4% DM, 90.7% OM, 11.9% CP, 47.3% NDF, 24.8% ADF, and 20.5% starch.

[b]Composition (DM basis): 96.7% OM, 13.6% CP, 19.6% NDF, 6.8% ADF, and 58.1% starch.

[c]Composition (DM basis): 94.0% OM, 40.2% CP, 28.2% NDF, 9.8% ADF, 4.1% ether extract, and 1.7% starch.

[d]Supplement consisted of (dietary DM) 0.92% ground barley, 0.83% canola meal, 1.04% calcium carbonate, 0.04% molasses, 0.09% salt, 0.02% feedlot premix (supplied per kilogram of dietary DM: 15 mg Cu, 65 mg Zn, 28 mg Mn, 0.7 mg I, 0.2 mg Co, 0.3 mg Se, 6,000 IU vitamin A, 600 IU vitamin D, and 47 IU vitamin E), 0.06% urea, 0.002% vitamin E (500,000 IU/kg), and 0.02% canola oil.

period was 17 days, comprising 10 days for adaptation and 7 days for data and sample collection.

On day 1, a bag containing 20 g of wet solid rumen contents, a diet bag containing 700 ml ruminal fluid, and 200 ml of warmed McDougall's buffer (11) were added to each fermenter. Ruminal liquid and solid were added to inoculate the feed with liquid- and particle-associated microbes. The McDougall's buffer was modified to contain 0.82% $NaH_2PO_4 + H_2O$ (wt/vol), 0.63% $NaHCO_3$ (wt/vol), and 0.03% $(NH_4)_2SO_4$ (wt/vol). The McDougall's buffer was continuously infused into the fermenters at a dilution rate of 2.9%/h during the entire experimental period. Solid rumen content bags were removed after 24 h of incubation and replaced with a diet bag. Thereafter, each fermenter contained two diet bags with one bag being removed daily and a new bag inserted. Thus, the incubation time for each bag in the fermenter was 48 h. During bag exchange, anaerobic conditions were maintained by the infusion of CO_2 into the fermenters. Fermented gases and accumulated effluents were collected into reusable 2 L gas-tight vinyl collection bags (Curity®; Covidien Ltd., Mansfield, MA, USA) that were attached to each of the effluent vessels and Erlenmeyer flasks (2 L), and production was measured daily.

Source of Inoculum

Solid and liquid ruminal contents were collected from different locations in the rumen (6 L per animal) at 2 h after morning feeding from three ruminal fistulated beef heifers (average, 698 ± 65.1 kg body weight). The heifers were fed a high-grain diet containing 15% barley silage, 82% barley grain, and 3% vitamin and mineral supplement (DM basis). The collected rumen contents were immediately filtered through four layers of cheesecloth, pooled, and pH recorded before dispensing into the fermenters.

Fermentation Characteristics and N Fraction

Fermenter media pH was measured during bag exchange using a calibrated pH meter (Orion model 260A, Fisher Scientific, Toronto, ON, Canada). During the sampling period, the effluent was collected for the analysis of VFA, NH_3-N, and soluble N. To determine VFA concentration, 5 ml of effluent was added to 1 ml of 25% metaphosphoric acid (HPO_3) (wt/vol) in screw-capped vials and stored at −20°C until analysis. The VFA concentration was determined using a gas chromatograph (model 5890, Hewlett-Packard Labs, Palo Alto, CA, USA), with a capillary column (1-μm phase thickness, 30 m × 0.32 mm i.d., Zebron ZB-FFAP, Phenomenex, Torrance, CA, USA), a flame ionization detector, and trans-2-butenoic acid as an internal standard. To measure NH_3-N concentration, 5 ml of effluent was added to 1 ml of 1% H_2SO_4 (wt/vol) in screw-capped vials and stored at −20°C until analysis using the modified Berthelot method (12). Daily VFA and NH_3-N production were calculated by multiplying the concentration of the fermentation end product in the effluent by the daily production of effluent.

Large and small peptide N were determined by measuring soluble N in the effluent using tungstic acid (TA) and trichloroacetic acid (TCA), as described by Winter et al. (13) and Li et al. (14). Four milliliters of effluent was mixed with 1 ml of 10% (wt/vol) sodium tungstate and 1 ml of 1.07 N sulfuric acid to measured TA soluble N (TA-N). TCA soluble N (TCA-N) was measured by adding 1 ml of 50% (wt/vol) TCA solution to 4 ml of effluent. All tubes were kept at 5°C for 4 h and then centrifuged at 9,000 × g for 15 min. All supernatants were collected for the analysis of TA-N and TCA-N using flash combustion and a thermal conductivity detector (Model 1500; Carlo Erba Instruments, Milan, Italy). The large peptide N concentration was calculated as the difference between TCA-N and TA-N, while the small peptide N (including amino acid; AA) concentration was calculated by the difference between TA-N and NH_3-N.

Nutrient Disappearance and Gas Production

Dry matter disappearance (DMD) was determined from day 11 to day 15 of the sampling period. After 48 h of incubation, the bags were removed, gently squeezed, and washed under running cold tap water until the water was clear. The bags were oven-dried at 55°C for 48 h and weighed to determine the DMD. The residue in the bags was removed, pooled by using the fermenter, and ground through a 1-mm sieve (standard model 4, Arthur Thomas Co., Philadelphia, PA, USA) for chemical analysis. The experimental diets and the residue from the bags were analyzed

for DM (method no. 930.15) and ash content (method no. 942.05) according to the AOAC (15). Neutral detergent fiber (NDF) concentration was determined according to the method described by Van Soest et al. (16) using heat-stable amylase and sodium sulfite and expressed inclusivity of residual ash. The concentration of acid detergent fiber (ADF) was determined according to method no. 973.18 (15). A combustion analyzer (NA 2100, Carlo Erba Instruments, Milan, Italy) was used for total *N* analysis (method no. 990.03; 15), and the CP was calculated as $N \times 6.25$. The starch was determined by enzymatic hydrolysis of α-linked glucose polymers as reported previously (17). The fat content was determined using ether extraction (Extraction Unit E-816, Büchi Labortechnik AG, Flawil, Switzerland; 15; method no. 920.39). Nutrient disappearance was calculated as the difference between nutrient concentration in the substrate before and after incubation.

Daily gas production (GP) was measured during bag exchange in the morning using a wet-type gas meter (Alexander-Wright, London, UK). From day 11 to day 15, a gas sample (20 ml) was collected from each bag using a 26-gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA) which is injected into evacuated 6.8-ml Exetainer vials (Labco Ltd., High Wycombe, Bucks, UK). The methane (CH_4) concentration in the gas samples was determined, as described by Avila-Stagno et al. (18), using a Varian CP-4900 mICRO Gas Chromatograph equipped with a GS-CarbonPLOT 30 m × 0.32 mm × 3 μm column and a thermal conductivity detector (Agilent Technologies Canada Inc., Mississauga, ON, Canada).

Microbial Protein Synthesis

Feed particle-associated (FPA) bacteria, feed particle-bound (FPB) bacteria, and effluent liquid-associated bacteria (LAB) were evaluated to determine microbial protein synthesis. The bacteria were labeled with ^{15}N, and on day 10 of the experiment, samples were collected from the effluent and substrate residue to determine the analysis of background ^{15}N. On day 11, $(^{15}NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, MO, USA; minimum ^{15}N enrichment 1 g/L) was added to the McDougall's buffer instead of $(NH_4)_2SO_4$ until the end of the sampling period, and sodium azide was added to effluent flasks to stop microbial activity (3 ml/flask per day; 0.1% wt/vol final concentration).

On days 16 and 17, 35 ml of effluent were sampled for LAB determination. The effluents were centrifuged at 20,000 × g at 4°C for 30 min, and the resulting pellets were washed using deionized water and centrifuged three times (20,000 × g, 30 min, 4°C). The pellets were suspended in distilled water and freeze-dried until N and ^{15}N analysis. FPA bacteria and FPB bacteria were measured by removing the bags from the fermenters and gently squeezing to expel excess liquids. Thereafter, bags were weighed and placed individually in plastic bags with 20 ml of phosphate buffer and were processed two times in a Stomacher 400 Circulator laboratory blender (Seward Medical Ltd., London, UK) for 1 min each time. The bags were then manually squeezed and washed two times with phosphate buffer. The squeezed and rinsed liquid (FPA fraction) was centrifuged at 500 × g at 4°C for 10 min to remove large feed particles, and the supernatant was centrifuged (20,000 × g, 30 min, 4°C) to isolate a bacterial pellet, which was washed three times as previously described. The pellets were suspended in distilled water and freeze-dried until N and ^{15}N analysis. The washed feed residues (FPB fraction) were oven-dried at 55°C for 48 h for DMD, and they were also ball ground (MM400; Retsch Inc., Newtown, PA, USA) for N and ^{15}N analysis. The flash combustion method (Model 1500; Carlo Erba Instruments, Milan, Italy) was used for total N determination. The ^{15}N enrichment was measured by ^{15}N continuous flow measurement using a combustion analyzer interfaced with a mass spectrometer (VG Isotech, Middlewich, UK).

Microbial Community

High-throughput sequencing techniques were used to evaluate FPA microbial communities. Thirty milligrams of the FPA samples were obtained for total DNA extraction and were analyzed with the QIAamp Fast DNA Stool Mini Kits (Qiagen, Hilden, Germany) as per the instructions provided by the manufacturer. Before the DNA extraction, the freeze-dried rumen samples were bead beat for 2 min at 50 Hz to break down any fiber particles that may block the DNA-binding column during DNA extraction. The concentration and purity of the DNA were checked using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNA samples were stored at −20°C until sequencing analysis. The PCR technique and DNA sequencing were completed following the method defined by Walters et al. (19). Amplification of the V4 region of the bacterial 16S rRNA gene was conducted using modified 515-F and 806-R primers. About 99% OTUs of Greengenes 13_8 from the 515F/806R region of the sequence database were used, and no difference in methanogens was found among treatments. A two-step PCR was followed to produce the 165 rRNA gene amplicons, which were then exposed to Illumina paired-end library preparation and cluster generation. The Illumina MiSeq instrument was used for sequencing (Illumina, Inc., San Diego, CA, USA). The R package DADA2 (Version 1.4) denoising method and QIIME2 were used for treating the raw data for gene sequencing according to Bolyen et al. (20). Primer sequences were removed, forward and reverse reads were truncated at 225 bp, and the read quality was checked using QIIME2. The number of OTUs (richness) and the Shannon index (diversity) were also calculated. Non-metric multidimensional scaling (NMDS) was carried out using the R packages vegan (Version 2.4.4) and phyloseq (Version 1.20.0) according to the Bray–Curtis similarity distances. Ruminal bacterial fold change at the genus level with a 5% threshold was considered using the R package Deseq2 (21).

Statistical Analysis

The data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA) as repeated measures according to a 2 × 2 factorial arrangement of treatments. The model included wheat DDGS, ROD extract, and their interaction as fixed effects, with the fermenter and the RUSICTEC apparatus as random effects. Sampling days were used as repeated measures for GP profiles, fermenter pH, VFA production and profiles, NH_3-N production, and DMD parameters. Several covariance structures, such as the compound symmetry, heterogeneous compound

symmetry, autoregressive, heterogeneous autoregressive, and unstructured, were carried out for repeated measures, and a low Akaike's information criterion value was chosen. Results were declared as least square means, and the significance was reported at $P \leq 0.05$ and a trend was reported at $0.05 < P \leq 0.10$, unless otherwise stated.

RESULTS

Fermentation Characteristics and N Fraction

Fermenter pH was higher ($P = 0.01$) with DDGS than the basal diet, with the supplementation of ROD extract having no effect (**Table 2**). Total VFA production and the molar proportion of acetate, propionate, and butyrate were not affected by the diets or supplementation with the ROD extract, except for a tendency for the butyrate to be higher ($P = 0.08$) with ROD extract supplementation; no interaction between the ROD extract and the diets was observed. Basal diet had a lower ($P = 0.01$) molar proportion of branched-chain VFA (BCVFA) and valerate but a higher ($P = 0.01$) proportion of caproate compared to the DDGS diet, with the supplementation of the ROD extract reducing ($P = 0.01$) caproate in both diets. The production of NH_3-N was higher ($P = 0.01$) with DDGS compared to the basal diet, with no observed effect of the ROD extract. The large peptide N was higher ($P = 0.01$) with DDGS compared to the basal diet, with the opposite outcome ($P = 0.02$) observed for the small peptide N fraction. No interactions were observed in the N fraction.

Nutrient Disappearance and Gas Production

There was no significant effect of DDGS and ROD extract on nutrient disappearance of DM, organic matter (OM), ADF, and CP (**Table 3**). In contrast, the NDF disappearance was increased ($P = 0.01$) by partially replacing barley grain with wheat DDGS. No interactions were observed except for a tendency ($P = 0.06$) of starch disappearance. The NDF disappearance was ($P = 0.10$) higher but starch disappearance was lower ($P = 0.06$) when supplementing ROD extract in the basal diet, but with no effect in the DDGS diet. No effects of DDGS or ROD extract supplementation on GP and CH_4 production (mg/d) were observed. There was a tendency for DDGS to have higher CH_4, expressed as percentage of gas ($P = 0.05$) and mg/g digested basis ($P = 0.08$), compared to the basal diet. No interactions between the basal diet and ROD were observed.

Microbial Protein Synthesis

The production of total microbial N and LAB was not affected by the inclusion of DDGS, whereas FPA production decreased ($P = 0.06$) and the FPB production increased ($P = 0.01$) with the addition of DDGS compared with the addition of basal diets (**Table 4**). The production of microbial N was not affected by supplemental ROD extract, and the efficiency of microbial N synthesis did not differ among treatments. No interactions between DDGS and ROD extract diets were observed.

Microbial Community

The number of OTUs (**Figure 1,** Observed) and the Shannon diversity index (**Figure 1**, Shannon) showed that the microbial community had little variation among treatments (variance in the mean of Shannon diversity index = 0.002, variance in the median of Shannon diversity index = 0.009). However, the addition of ROD extract to the DDGS diet resulted in the highest diversity in bacterial species (Median Shannon index = 3.7), while the lowest diversity (highest homogeneity) was observed with the basal diet (Median Shannon = 3.5). Similarly, the NMDS plot depicted in **Figure 2** showed no separation or absence of specific clustering of FPA microbial communities among treatments, indicating a high similarity between samples taken from the various diets in terms of the identified bacterial genera.

Taxonomic analysis, as shown in **Figure 3**, showed that the phylum Firmicutes exhibited the highest abundance (abundance = 62.7) among all other phyla irrespective of the diet, followed by the phylum Bacteroidetes (abundance = 24.6). Other phyla existed with very low abundance (range of 3.9–0), indicating that the addition of the ROD extract had no effect on the relative abundance of the bacteria at the phylum level. At the genus level, *Prevotella* (median = 26.4) and *Fibrobacter* (median = 5) were the most abundant genera in the basal diet; however, *Treponema* became the most abundant genus (**Figure 4**) with the addition of the ROD extract. The dominant genera associated with the DDGS diet were *Acidaminococcus* (median = 4.9), *Megasphaera* (median = 4.8), *Shuttleworthia* (median = 18.2), and *Lactobacillus* (median = 6.8). However, *Selenomonas* (median = 27.9), and *Schwartzia* (median = 4.2) were the dominant genera when the ROD extract was added to the DDGS diet.

The log2 fold change analysis indicated that the majority (97.3%) of the microbial community that differed ($P = 0.05$) between the basal and DDGS diets were upregulated while only five (2.6%) were downregulated (**Figure 5**). The upregulation was most prevalent in *Prevotella* (phylum Bacteroidetes), while *Selenomonas* (phylum Firmicutes) showed the highest downregulation. The addition of ROD extract resulted in changes in the microbial community in both the basal or DDGS diets, although the impact was greatest in the DDGS diet. Supplementation of the ROD extract in the DDGS diet increased all genera that belonged to the phylum Firmicutes (especially genera *Selenomonas*) but decreased the genus *Prevotella*, which belongs to phylum Bacteroidia. Adding ROD extract to the basal diet resulted in the upregulation of *Fibrobacter* (phylum Fibrobacteres), whereas *Shuttleworthia* (phylum Firmicutes) was the most downregulated genus.

DISCUSSION

Effects of Wheat DDGS

The wheat DDGS diet exhibited higher rumen pH than the basal diet, which is likely reflective of its lower starch content. Increasing dietary starch concentration is well-established to have a negative effect on rumen pH (22). Relative change between pH and VFA concentration appeared to be similar with a 2% increase in pH and a 2% decrease in

TABLE 2 | Effect of wheat dried distillers grains with solubles (DDGS) and the red osier dogwood (ROD) extract on fermentation and nitrogen fraction in RUSITEC.

Item	Basal[a]		DDGS[a]			*P*-value		
	–ROD	+ROD	–ROD	+ROD	SEM	DDGS	ROD	Inter
pH	5.74	5.75	5.90	5.90	0.02	0.01	0.95	0.99
Total VFA, m*M*/d	45.6	44.0	44.7	43.5	1.42	0.63	0.35	0.89
VFA, mol/100 mol								
Acetate (A)	27.8	28.9	29.0	28.9	0.33	0.10	0.19	0.10
Propionate (P)	41.2	39.9	40.1	39.6	0.58	0.25	0.49	0.15
Butyrate	20.1	21.0	19.8	20.5	0.42	0.39	0.08	0.72
BCVFA	2.1	2.2	2.3	2.3	0.04	0.01	0.42	0.42
Valerate	5.8	5.2	6.8	6.9	0.30	0.01	0.50	0.28
Caproate	3.3	2.6	2.0	1.8	0.14	0.01	0.01	0.10
A:P	0.67	0.72	0.72	0.73	0.02	0.06	0.15	0.08
NH_3 N production, mg/d	54.7	54.6	91.3	92.0	2.10	0.01	0.87	0.88
N fraction, mg/100 ml								
Large peptide	22.2	22.9	15.1	16.5	1.06	0.01	0.33	0.75
Small peptide	13.2	11.8	15.4	14.5	0.97	0.02	0.25	0.83
NH_3-N	8.2	8.0	13.5	13.4	0.33	0.01	0.79	0.94

SEM, standard error of the mean; Inter, interaction between DDGS and ROD; VFA, volatile fatty acid; BCVFA, branched-chain VFA (isobutyrate + isovalerate); DM, dry matter; N, nitrogen; NH_3-N, ammonia, N.

[a]The diet consisted of barley silage, barley concentrate, and wheat DDGS in the ratios of 10:90:0 and 10:65:25, respectively, for the basal and DDGS diets (DM basis); the ROD extract was added with 0 (–ROD) or 1% dietary DM (+ROD).

TABLE 3 | Effect of wheat DDGS and the ROD extract on nutrient disappearance and gas production (GP) in RUSITEC.

Item	Basal[a]		DDGS[a]			*P*-value		
	–ROD	+ROD	–ROD	+ROD	SEM	DDGS	ROD	Inter
Nutrient disappearance, %								
DM	76.3	77.5	77.3	77.5	0.69	0.43	0.35	0.45
OM	77.5	78.6	77.8	77.9	0.71	0.83	0.40	0.52
NDF	24.5	27.6	40.8	40.2	1.07	0.01	0.26	0.10
ADF	22.5	25.3	26.5	26.2	1.46	0.76	0.40	0.31
Starch	90.8	88.9	88.4	87.2	0.81	0.19	0.21	0.06
CP	82.5	83.1	82.5	82.1	1.06	0.64	0.97	0.65
Gas production								
GP, L/d	1.76	1.75	1.70	1.74	0.07	0.55	0.87	0.73
GP, ml/g DM digested	230.6	223.1	218.8	221.4	7.61	0.39	0.75	0.52
CH_4, % of gas	1.95	2.11	1.75	1.79	0.12	0.05	0.42	0.61
CH_4, mg/d	34.4	37.4	30.1	31.1	3.07	0.11	0.52	0.73
CH_4, mg/g DM digested	2.93	3.09	2.52	2.59	0.25	0.08	0.65	0.83

SEM, standard error of the mean; Inter, interaction between DDGS and ROD; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; CH_4, methane; GP, gas production.

[a]The diet consisted of barley silage, barley concentrate, and wheat DDGS in the ratios of 10:90:0 and 10:65:25, respectively, for the basal and DDGS diets (DM basis); ROD extract was added with 0 (–ROD) or 1% dietary DM (+ROD).

VFA concentration, indicating that the change in pH was primarily associated with decreased total VFA production. The increased NDF disappearance of the DDGS diets could be explained by a large fraction of digestible NDF in wheat DDGS (14) as well as the higher fermenter pH observed in the DDGS diets. Rumen cellulolytic bacteria are sensitive to rumen pH and adversely impacted at pH <6.0 (23), with pH of 5.8, 5.6, and 5.2 associated with mild, moderate, and severe subacute rumen acidosis, respectively (24).

In the rumen, the end products of feed substrates include VFA, gas (CH_4), and microbial biomass. The production of VFAs plays an important role in supplying the animal with the energy needed for vital physiological processes (25). The main factors affecting total VFA production in the rumen are diet composition and the digestibility and fermentability of feedstuffs

TABLE 4 | Effect of wheat DDGS and the ROD extract on microbial N synthesis in RUSITEC.

Item	Basal[a]		DDGS[a]			*P* value		
	–ROD	+ROD	–ROD	+ROD	SEM	DDGS	ROD	Inter
Microbial N, mg/d								
Total	81.4	80.2	84.4	86.6	5.74	0.38	0.92	0.72
LAB	66.8	64.9	71.0	71.2	5.20	0.36	0.87	0.83
FPA	12.1	12.7	9.7	11.0	1.10	0.06	0.38	0.72
FPB	2.3	2.8	4.1	4.3	0.16	0.01	0.12	0.36
EMPS, mg N/g OM digested	10.2	10.0	10.8	11.2	0.70	0.18	0.82	0.61

SEM, standard error of the mean; Inter, interaction between DDGS and ROD; N, nitrogen; LAB, liquid-associated bacteria; FPA, feed particle-associated bacteria; FPB, feed particle-bound bacteria; EMPS, efficiency of bacterial N synthesis; OM, organic matter.

[a]*The diet consisted of barley silage, barley concentrate, and wheat DDGS in the ratios of 10:90:0 and 10:65:25, respectively, for the basal and DDGS diets (DM basis); ROD extract was added with 0 (–ROD) or 1% dietary DM (+ROD).*

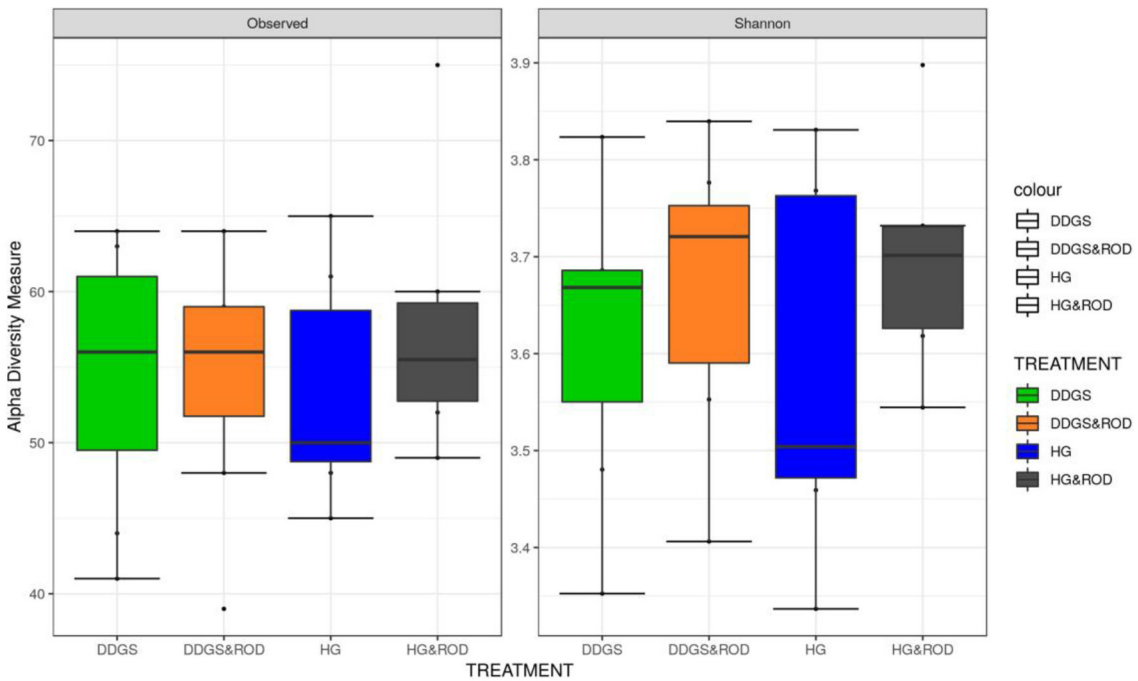

FIGURE 1 | Box plots of the number of operational taxonomic units (OTUs) and the Shannon diversity index for feed particle-associated (FPA) samples by treatment. Treatments were as follows: wheat dried distillers grains with solubles (DDGS) diet without adding red osier dogwood (ROD) extract; DDGS&ROD (wheat DDGS diet with ROD extract); HG (basal diet without adding ROD extract); and HG&ROD (basal diet with ROD extract).

(26). The lack of difference between basal and DDGS diets in total VFA production and OM disappearance suggested that the two diets had similar nutrient fermentability. Rumen VFA profiles and fermentation patterns (i.e., acetate to propionate [A:P] ratio) in cattle offered diets containing wheat DDGS vary, likely depending on the composition and the proportion of wheat DDGS in the diets (27–29). A higher A:P ratio with DDGS than the basal diet was also observed by Beliveau and McKinnon (29), who reported that the substitution of wheat DDGS from 0%, 7%, 14–21% of dietary DM for barley grain increased the A:P ratio due to increased molar proportion of acetate and decreased propionate proportion. In the present study, however, the trend of increased acetate concentration and the A:P ratio in the DDGS compared to those of the basal diet was lower than expected, given the higher rate of NDF disappearance. Additionally, increased BCVFA apparent with the DDGS diet likely resulted from increased ruminal protein degradation associated with a high dietary CP content. The formation of BCVFA occurs during protein degradation as a result of branched-chain AA deamination (30). The higher valerate concentration with DDGS diets is in agreement with another study on RUSITEC using wheat DDGS with a similar treatment design but with the addition of a plant essential oil [cinnamaldehyde; (28)]. This may be explained by the higher

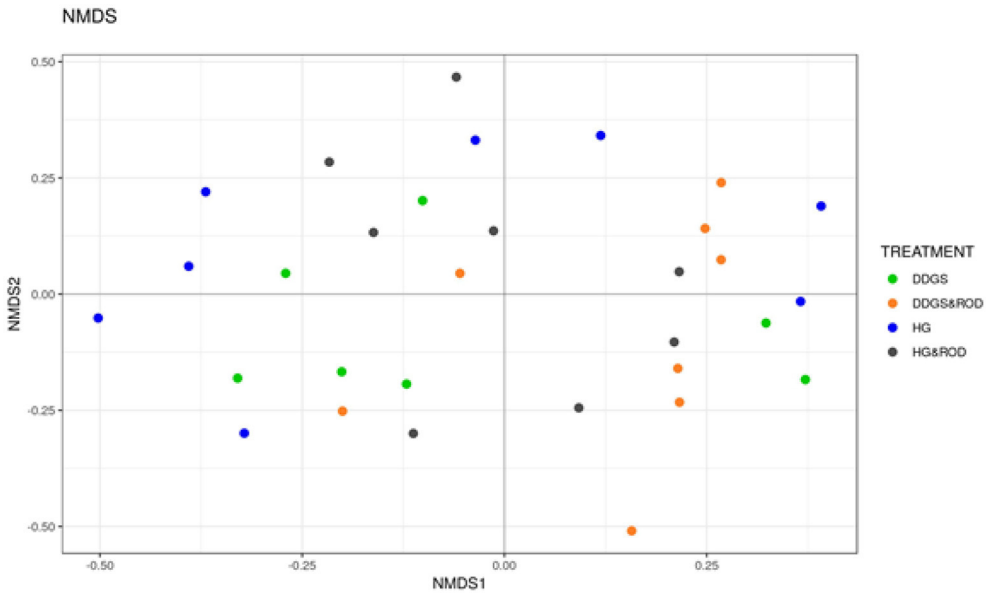

FIGURE 2 | Non-metric multidimensional scaling (NMDS) plots of the Bray–Curtis dissimilarities for FPA samples by treatment. Treatments were as follows: DDGS diet without adding the ROD extract; DDGS&ROD (wheat DDGS diet with ROD extract); HG (basal diet without adding ROD extract); and HG&ROD (basal diet with ROD extract).

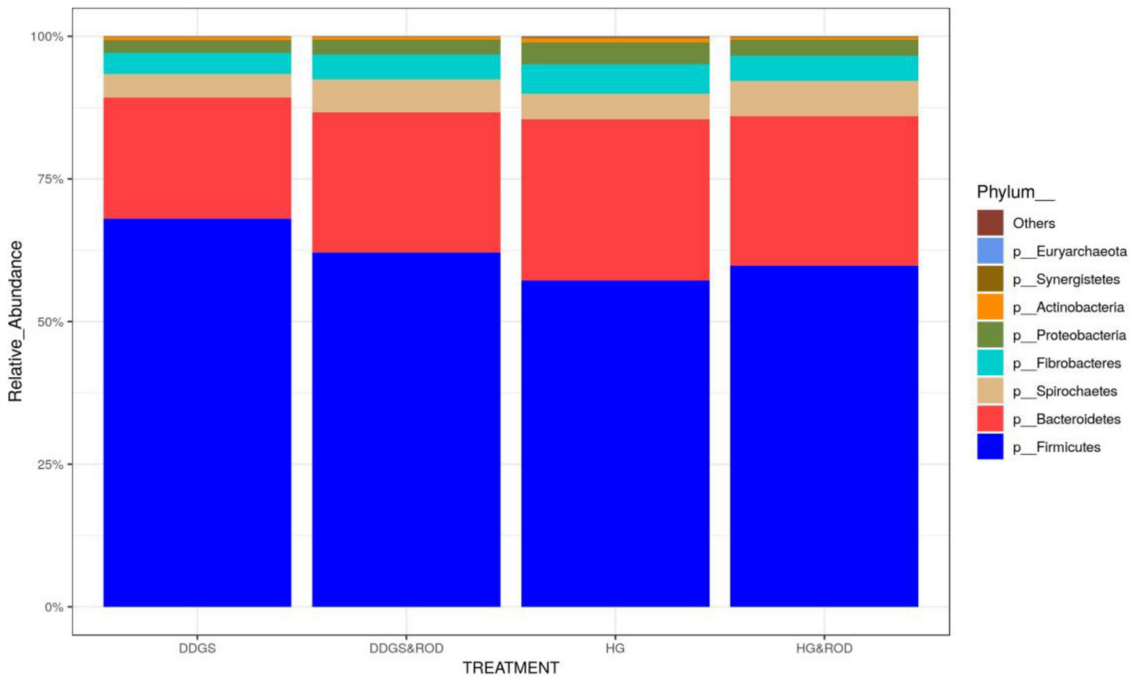

FIGURE 3 | Effect of different treatments on the relative abundance (RA) of the ruminal microbial community at the phylum level. Treatments were as follows: wheat DDGS diet without adding the ROD extract; DDGS&ROD (wheat DDGS diet with ROD extract); HG (basal diet without adding ROD extract); and HG&ROD (basal diet with ROD extract).

soluble fraction (e.g., potentially high in sugars) in wheat DDGS than the parent material (31), leading to an increase in the concentration of valerate in the rumen (32).

The similar disappearance of DM and OM is consistent with the absence of differences in the disappearance of starch and CP between basal and DDGS diets. Although the NDF disappearance

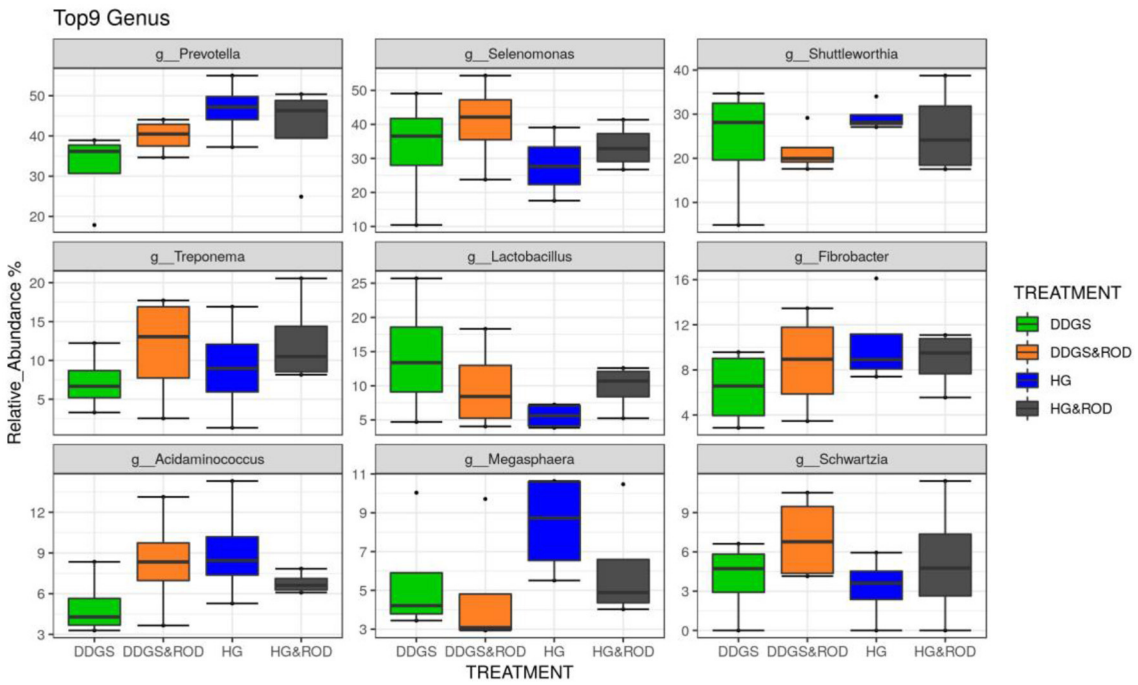

FIGURE 4 | Effect of different treatments on the RA of the ruminal microbial community at the genus level. Treatments were as follows: wheat DDGS diet without adding the ROD extract; DDGS&ROD (wheat DDGS diet with ROD extract); HG (basal diet without adding ROD extract); and HG&ROD (basal diet with ROD extract).

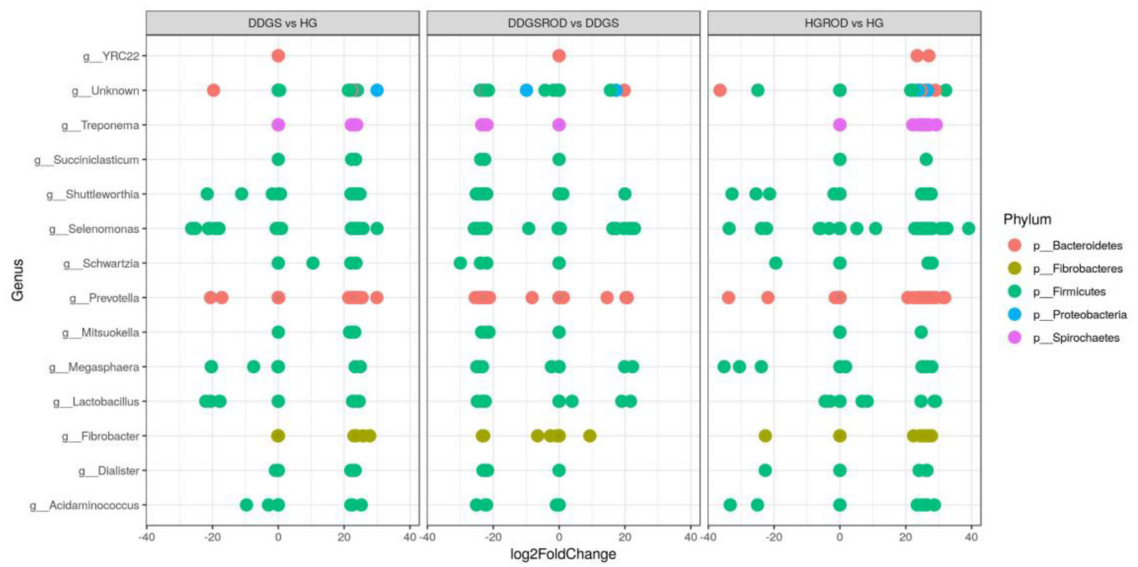

FIGURE 5 | Relative changes (log2 fold; $P < 0.05$) of ruminal bacteria by DDGS vs. HG, DDGS&ROD vs. DDGS, and HG&ROD vs. HG at the genus level. DDGSDDGS diet without adding the ROD extract; DDGS&ROD (wheat DDGS diet with ROD extract); HG (basal diet without adding the ROD extract); HG&ROD (basal diet with the ROD extract).

of the DDGS diet was higher than that of the basal diet, its quantitative contribution to the DM disappearance was smaller compared with starch and CP. The effect of adding wheat DDGS in barley grain-based diet on rumen DM digestibility is inconsistent in the literature (14, 28, 33). The discrepancy among studies may be due to the variation in the chemical composition of wheat DDGS, experimental conditions (14), and starch digestibility in grain vs. NDF digestibility of DDGS (33). Nevertheless, the ruminal NDF digestibility of wheat DDGS has been reported to be higher than that of feed grain or silage

(33, 34) owing to the high hemicellulose in DDGS, which is more digestible than cellulose. The CP disappearance was high (>82%) in the current study and was not affected by substituting wheat DDGS in the barley grain diet, suggesting that the NDF insoluble N was low. Furthermore, the higher small peptide and NH_3-N concentrations with the DDGS diet reflected the higher CP content and is in agreement with a previous study evaluating the effect of partly replacing barley grain with wheat DDGS in a high-grain diet (28).

The production of CH_4 has a negative impact on the energy efficiency in ruminants and represents a loss of dietary energy ranging from 2 to 12% of gross energy intake (35). Supplementation of diets with lipids that are not protected from ruminal digestion is one strategy recognized to lower enteric CH_4 emissions (36), and the reduction in CH_4 by adding wheat DDGS in the place of barley grain is hypothesized due to a higher fat contribution from the DDGS. This has been reported by McGinn et al. (37), who substituted 35% of corn DDGS for barley grain in the diet of growing beef cattle, leading to a 16% (units) reduction in enteric CH_4 production, which was attributed to the increased fat content (5.1%) of the diet. Some poly-unsaturated fatty acids in corn DDGS oil (especially linolenic acid) have been reported to specifically inhibit methanogenesis (38). Dugan et al. (39) reported that increasing wheat DDGS inclusion in beef cattle diets increased the percentage of linoleic acid from 51 to 56% of total fatty acids.

Adding wheat DDGS to barley-based diets has been reported to increase microbial protein production associated with improved OM digestibility (14, 28). The failure to increase microbial protein production with the DDGS vs. basal diets could be explained by the similar, but high, OM digestibility between basal and DDGS diets, thus denoting that energy is not be a limiting factor in microbial protein synthesis in the present study.

Although the ruminal microbial ecosystem can be affected by numerous factors, such as animal species, sex, stress, and environment (40), the most important factor is the type of diet (41). Henderson et al. (42) indicated that the diversity of the ruminal microbial community could be enhanced by diet complexity. In the current study, the diversity of the microbial community was not different among diets, which is in agreement with the study by Li et al. (28), who reported similar copy numbers of 16S rDNA of *Fibrobacter* and *Prevotella* between basal and wheat DDGS diets. However, when adding a plant essential oil (cinnamaldehyde) to either diet, copy numbers of *Fibrobacter* and *Prevotella* decreased (28). *Prevotella* exhibited upregulation while *Selenomonas* exhibited downregulation with the DDGS diet, indicating that the copy numbers of *Prevotella* may have responded to the higher CP content of the wheat DDGS. This result is in agreement with that from the study of Callaway et al. (43), who indicated that the *Prevotella* population could be enhanced by high CP concentrations. While *Selenomonas* is responsible for the fermentation of monosaccharides and disaccharides, as well as the production of propionate, the decrease in *Selenomonas* population explains the similarity in the propionate concentration.

Effects of ROD Extract

It is well-established that supplementing feed additives can alter individual VFA profiles and fermentation patterns (5, 9). For example, it is well-known that feeding monensin to ruminants increases propionate production and decreases the A:P ratio, thus improving energy efficiency (5). The trend of increased acetate concentration and the A:P ratio due to the addition of the ROD extract to the basal diet in the present study is in agreement with the previous batch culture study (5). These findings suggested that starch disappearance has decreased and the NDF disappearance has increased because of adding ROD, which did occur in the current study. The reduction in starch digestibility because of adding the ROD extract was consistent with the changes of the microbial community at the genus level. *Treponema* was observed to be the most abundant genus, replacing the genera *Prevotella* and *Fibrobacter*. *Prevotella* at the genus level is usually dominant in the rumen microbial community associated with high-grain diets (44), as they are able to utilize various nutrients such as starch, proteins, and non-cellulosic polysaccharides (45).

Contrary to the previous batch culture study, where DMD and VFA production decreased by adding ROD to a high-grain diet (5), the disappearances of DM and OM and total VFA production were not affected by adding the ROD extract in the present study. The discrepancy between the two studies may be related to variations in the concentration of phenolic compounds between raw ROD and ROD extract, diet composition, or the *in vitro* technique used. Singh et al. (46) reported that the response of total VFA production to plant polyphenolic extracts varied with the original plant species and the level of inclusion of the extract. In the batch culture study conducted by Wei et al. (5), the fermentation period was shorter (i.e., 24 h), whereas, in the current study using RUSITEC, the data and samples were collected for 7 days followed by 10 days of microbial adaptation, thus indicating that the rumen microorganisms may have adapted to the treatments. Furthermore, the different levels of inclusion of phenolics used and their profiles may also lead to the variation between studies. In the previous study (5), the total amount of phenol used was 31, 62, or 124 mg/L, compared to 33 mg/L of fermentation media in the present study. The phenol composition appeared to be different between raw ROD and ROD extract; the phenolic compounds were identified as having a higher number of active compounds (gallic acid, methyl gallate, catechin, epicatechin, rutin, ellagic acid, and quercetin) in raw ROD compared to the ROD extract (gallic acid, ellagic acid, rutin, quercetin malonyl glucoside, and quercetin). These results suggest that, similar to raw ROD, adding ROD extract to a high-grain diet can impact rumen fermentation and microbial activity, which may vary with the phenolic compound profile. In the present study, the addition of the ROD extract led to a change in the dominant genera bacteria in both the basal and DDGS diets, indicating selective promotion or suppression of specific bacteria performed by the ROD extract.

Phenolics have both antioxidant and antimicrobial properties (2, 4); however, studies regarding the effect of these compounds on rumen fermentation and microbial activity are scarce (47).

Furthermore, no other studies have assessed the effect of the ROD extract on the ruminal microbial community. No effect of adding the ROD extract on the relative abundance of bacteria at the phylum level is consistent with the absence of differences in GP, nutrient disappearance, and microbial protein synthesis. However, at the genus level, changes in the microbial community were observed, and it revealed altered microbial activity with the addition of the ROD extract. Such activity may not be necessary to significantly increase rumen nutrient degradation under present experimental conditions, especially with highly fermentable carbohydrates that are present in high-grain diets. Furthermore, the rumen is recognized to be an anaerobic environment; however, some oxygen may enter through feeds causing oxidative stress. Therefore, the ROD extract, which has antioxidants that reduce ruminal oxidative stress, may improve rumen function. However, the absence of effects of feeding the ROD extract on ruminal pH and VFA profiles suggested that low oxidant stress occurred in the rumen under the RUSITEC conditions of the present study.

Interaction of DDGS Inclusion and ROD Extract

A number of interaction trends were observed between dietary DDGS and ROD extract supplementation, which were consistent with the study of Li et al. (28), who reported that *in vitro* rumen NDF digestibility and protozoan counts responded differently to the supplementation of plant essential oil (cinnamaldehyde) when barley grain was partly replaced with wheat DDGS in a high-grain diet. Both plant essential oil and phenols, plant-derived secondary active components, have demonstrated antimicrobial and antioxidant activity in livestock animals (28, 46, 47). The interaction of DDGS with the ROD extract and its effect on the A:P ratio and the disappearances of NDF and starch were expected. A previous study observed that increasing the substitution of ROD for barley silage in a high-grain diet decreased the *in vitro* propionate concentration and DMD but linearly increased the A:P ratio, suggesting decreased that the starch digestibility (5). In the present study, the basal diet contained higher starch and thus would be more likely to be affected by adding ROD extract. Concurring with the previous study, adding ROD extract increased the NDF disappearance of the basal diet with no effect in the DDGS diet, while reduced starch disappearance was more pronounced with the basal diet compared with the DDGS diet. Decreased rumen starch digestion with the addition of the ROD extract may potentially help alleviate rumen acidosis in beef cattle that are fed high-grain rations. Moreover, Li et al. (28) suggested that wheat DDGS may contain some exogenous components, such as yeast cells, to stimulate rumen microbial activity, thus possibly offsetting the ROD extract activity in diets containing DDGS, which would explain the absence of the effect of DDGS when the ROD extract was added.

The lack of interaction between DDGS and the ROD extract supplementation on protein degradability was somewhat unexpected, as Wei et al. (6) reported that beef heifers that were fed ROD with high phenol concentrations had lower rumen NH_3-N concentration. The authors suggested that the supplementation of ROD to beef cattle that are fed high-grain diets may improve microbial protein synthesis or induce an increase in rumen bypass protein due to the protein-binding capacity of ROD phenols. Therefore, differences in the CP content between the basal and DDGS diets (basal vs. DDGS; 13.9 vs. 19.6% CP) were expected to result in differences in NH_3-N concentration, microbial protein synthesis, or CP degradability. Gomaa et al. (48) reported that the *in situ* rumen degradation rate linearly decreased for silage protein, but it was not changed for grain protein when heifers were fed diets containing ROD. Wei et al. (5) suggested that phenols in ROD have a greater protein-binding capacity with soluble protein than with insoluble protein. The percentage of soluble protein was 3.5 and 6.4% of DM, respectively, for wheat grain and wheat DDGS (33).

CONCLUSIONS

Replacing barley grain with wheat DDGS in a high-grain diet increased fermenter pH and molar proportion of BCVFA and switched the fermentation pattern to higher acetate production due to increased disappearance of NDF. The DMD and microbial protein production were not affected, whereas CH_4 production tended to decrease with the inclusion of wheat DDGS. In conclusion, the substitution of wheat DDGS for barley grain at 25% of DM in high-grain diets potentially mitigates CH_4 emissions. The supplementation of ROD extract at the rate of 1% of diet DM did not affect the GP; total VFA production; the disappearance of DM, OM, and CP; and microbial protein synthesis but increased the NDF disappearance, decreased starch disappearance, and increased the A:P ratio of the basal diet without DDGS. The decreased starch fermentability and the increased NDF disappearance as a result of adding the ROD extract suggest that the ROD extract may be used to improve rumen acidosis and fiber utilization for beef cattle that are fed with high-grain diets.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA693324.

ETHICS STATEMENT

The animal study was reviewed and approved by Lethbridge Research and Development Centre Institutional Animal Care and Use Committee (49).

AUTHOR CONTRIBUTIONS

WG, AS, TR, LJ, MS, EM, KO, LC, and WY: conceptualization, methodology, writing, reviewing, and editing. WG, AS,

and LJ: formal analysis. WG: preparation and writing of the original draft. WY: supervision. WY and LC: project administration. WY, LC, EM, and KO: funding acquisition. All authors contributed to the article and approved the submitted version.

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Feeding Sheep Cobalt and Oregano Essential Oil Alone or in Combination on Ruminal Nutrient Digestibility, Fermentation, and Fiber Digestion Combined With Scanning Electron Microscopy

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The feeding of Co lactate (Co), an essential oil blend (EO; oregano), or a combination of Co and EO (EOC) may improve nutrient digestion of corn silage-based rations. In four separate studies, Co, EO, or EOC was fed at 0, 4, and 7 g/days to nine rumen fistulated rams arranged in a replicated 3 × 3 Latin square design. The fourth study evaluated the carrier at 0, 4, and 7 g/day. In each ram, fresh ensiled corn silage, leaf, and husk were placed in individual nylon bags inserted through the ruminal cannula and removed after 48 h. Rams fed increasing carrier rates demonstrated similar ($P > 0.10$) nutrient digestibilities and ruminal pH and volatile fatty acid concentrations. Feeding Co at 4 and 7 g/day increased ($P < 0.05$) digestibility of DM (59.4, 63.9, and 62.4% for 0, 4, and 7 g/day, respectively), NDF (59.4, 63.9, and 62.4%), and hemicellulose (HC; 56.2, 63.6, and 65.9%) compared with rams fed 0 g/day, while CP digestibility (46.4, 49.9, and 57.8%) was improved ($P < 0.05$) in rams fed 7 g/day compared with those fed 0 and 4 g/day. Rams fed 4 g/day EO digested greater ($P < 0.05$) HC (64.1, 71.4, and 69.1%) than rams fed 0 g/day, while rams fed 7 g/day were intermediate and similar ($P > 0.10$). Rams fed the EOC combination at 4 and 7 g/day demonstrated greater ($P < 0.05$) digestibilities of DM (57.7, 60.0, and 60.0%), NDF (21.4, 28.8, and 27.7%), and ADF (24.3, 33.3, and 34.4%) than rams fed 0 g/day. The SEM and SM techniques visually demonstrated minor evidence of husk and leaf digestibility in rams across the three experiments when fed 0 g/day of Co, EO, or EOC; rams fed 4 g/day of Co, EO, or EOC exhibited varying visual signs of leaf digestion with some palisade tissue, spongy tissue, and whole vein structure remaining, while in rams fed 7 g/day, only the vein structure remained. Results demonstrated that feeding Co, EO, or EOC at 4 or 7 g/day enhanced ruminal nutrient digestion and fermentation parameters, which was visually confirmed via SEM and SM.

Keywords: cobalt, essential oil, digestibility, fiber structure, sheep, scanning electron microscopy

INTRODUCTION

China has a large agricultural economy with nearly 400 million ha of grassland, but overgrazing has resulted in significant amounts of confinement feeding using poorly digestible mature forages and crop residues, i.e., corn stalks, straw, etc. Various technologies exist to enhance nutrient and fiber digestibility among forage and crop residues having a wide digestibility range, but two promising technologies are feeding cobalt lactate (Co) and essential oils (EOs).

Co has been shown to improve fiber digestion, both in *in vitro* (1, 2) and *in vivo* studies (3–5). Oregano oil is a natural-plant-extracted EO that has a Generally Regarded as Safe (GRAS) status for livestock consumption (6–8). Oregano is reported to have antifungal, antiviral, and Gram-positive and Gram-negative bactericidal and bacteriostatic effects (8, 9). Initial EO ruminal fermentation studies demonstrated an inhibition of gas production (10, 11), and later studies reported altered ruminal fermentation by improving protein metabolism, volatile fatty acid (VFA) production, fiber digestion, and microbial community alteration (8, 12, 13), which altered milk composition (14–16).

A number of research studies conducted by our team has demonstrated improved calf growth when including EO to the milk replacer fed to calves or a blend of Co and EO (Rum-A-Fresh International or Stay Strong Domestically, Ralco Inc., Marshall, MN) fed in the calf starter or ration to growing calves and bulls (7, 17–19) and lactating dairy cows (20). These *in vitro* and *in vivo* trials demonstrated that feeding the Co and EO blend shifted ruminal fermentation to more total VFA and molar propionate percentages while reducing methane emissions and altering the microbial community, which led to improved dry matter, protein, and fiber digestion. However, in order to determine the exact mechanism of action, the individual components along with the combination were evaluated in a mechanistic study to identify specific responses to each component and identify the potential synergistic mechanisms.

Our current research program is focused on improving forage and crop residue utilization for enhancing ruminal N and fiber (energy) utilization by sheep. Most Co and EO (oregano) research projects have used fixed inclusion rates, and little is known regarding responses based on varying the inclusion rates combined with possible synergistic mechanisms among these technologies. For example, Froehlich et al. (7) reported that EO feeding rates used in an earlier work may have been too high to elucidate growth responses, and those high EO feeding rates may have been detrimental to growth performance. Froehlich et al. (7) demonstrated improved growth rates when feeding the lowest (2.5 g/day) EO inclusion rate compared to higher EO feeding rates. The study hypothesis was that sheep fed Co, EO, and/or EOC (combination) could potentially benefit alone or in combination from application of these fiber-digesting-enhancing technologies for improving ruminal nutrient digestion and fermentation, and these ruminal responses may be influenced by feeding rate. Therefore, the experimental objectives were as follows: (1) to measure nutrient digestion and ruminal fermentation characteristics in rams when fed increasing rates of Co and oregano EO alone or in combination (EOC) and (2) to elucidate the beneficial effects of feeding these additives alone or in combination for improving ruminal nutrient (fiber) digestibility. This study was designed to be a mechanism study in contrast to an animal performance study. The novel and unique use of a scanning electron microscope (SEM) and stereoscopic microscopy (SM) in digestion studies has not been conducted before, to our knowledge, which contributes to this study's uniqueness. The use of SEM and SM techniques does not allow for analyzing large numbers of samples from animal performance trials. However, the use of SEM and SM allows for visual observations to confirm findings of digestion.

Abbreviations: Co, cobalt lactate; EO, essential oils; EOC, essential oils and cobalt; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; HC, hemicellulose; SEM, scanning electron microscopy; SM, steroscopic microscopy; FDA, Food and Drug Administration; VFA, volatile fatty acids; TVFA, total volatile fatty acids; CARR, carrier; TMR, total mixed ration; NH_3-N, ammonia nitrogen; DMD, dry matter digestibility; CPD, crude protein digestibility; NDFD, neutral detergent fiber digestibility; ADFD, acid detergent fiber digestibility; Y_{ijk}, dependent variable; T_i, treatment; P_j, period; R_k, ram; e_{ijk}, radom error.

MATERIALS AND METHODS

Experimental Treatments

All experiments were conducted according to the Standards for the Care and Use of Research Animals (21) at Lintao Dairy and Animal Research Farm of Gansu Agricultural University, China or at Gansu Agricultural University, Lanzhou, China. All procedures were approved by the Institutional Animal Care and Use Committee of Gansu Agricultural University. The three additives evaluated as individual experiments were the following: (1) Co, 0.1425% cobalt lactate + 97% carrier + 2.8575% herbal package; (2) oregano EO, 1.13% essential oil (primarily microfused oregano oil particles <5 μm in diameter and uniform in size + small amount of olive oil) + 97% carrier + 1.87% herbal package; and (3) EOC, 0.1425% cobalt lactate + 1.13% oregano essential oil + 97% carrier + 1.7275% herbal package. The herbal carrier (CARR) product was composed of 75% zeolite (clinoptilolite) + 12% limestone + 10% diatomaceous earth and a herbal package that included small amounts of lactic acid, kelp, roughage products, chicory root, red pepper, fenugreek flavor extract, anise oil, cloves, saccharin sodium, and guar gum. The three additives and CARR were manufactured, packaged, shipped, and donated for the research project by the Animal Health Division of Ralco, Inc. (Marshall, MN, USA). Four independent similar experiments (three additives and CARR) were conducted evaluating the three feed additives (Co, EO, and EOC) and CARR, which were fed at 0 (control), 4, and 7 g/day using a thrice replicated 3 × 3 Lain square design having 21-day periods, with the last 10 days of each experimental period including incubation of corn silage, husk, and leaves samples placed in individual nylon bags (i.e., *in situ*). The 7 g/day inclusion rate was based on a company (Ralco, Inc.)-recommended EOC feeding rate of 28 g/day for lactating dairy cows scaled down to a sheep's average body weight. A misunderstanding/miscommunication between all coauthors on

TABLE 1 | Ingredient composition and nutrient concentration of the experimental diet.

Ingredient	(% As Is)	Nutrient	% of DM
Corn straw silage	72.96	DM (%)	36.2
Corn	14.41	DE (MJ/kg)	14.1
Bran	4.00	ME (MJ/kg)	11.5
Rapeseed meal	3.59	CP (%)	14.7
Cottonseed meal	3.86	Ca (%)	0.76
1% Premix	0.29	P (%)	0.65
Calcium bicarbonate	0.31		
Mineral meal	0.31		
Salt	0.28		
Total	100.00		

the experimental design resulted in the 0 g/day feeding rate for the Co, EO, and EOC experiments not including the product carrier (CARR) in 0 g/day treatment; therefore, the CARR (fourth) experiment was conducted at a later date at Gansu Agricultural University, Lanzhou, China to demonstrate that the CARR was not influencing the experimental results in the first three experiments because repeating the entire experimental series was not possible.

The total mixed ration (TMR) was formulated to meet the nutrient requirements of a ram weighing 55 kg and gaining 50 g/day according to the mutton sheep breeding standards (NY/T816-2004) of the Agricultural Industry Standard of the People's Republic of China. The forage to concentrate ratio was ∼70:30 (1.6 kg/day maize straw silage and concentrate supplement). The ingredient and nutrient compositions of the TMR are given in **Table 1**. The TMR was mixed daily (Animal Husbandry Machinery Co., Ltd., Hebei, China) and fed twice daily at 9:00 A.M. and 5:00 P.M. with *ad libitum* access to water.

Animals and Management

Three months prior to the start of the experiment, nine healthy German Merino sheep ♂ × Lintao local hybrid ♀ second-generation brucellosis-negative rams were surgically fitted with a permanent rumen fistula (China Agricultural University, Beijing, China). After surgery, the rams were fed a supplement formulated for the prevention of parasites and diseases while recovering and maintaining body condition during the postsurgical healing period. Rams averaged 53.7 kg body weight (BW) with an average rectal temperature of 39.8°C during postsurgical recovery. At the initiation of the experiment, the fistulated rams were blocked by BW and age and randomly assigned to one of three Latin squares having three treatments of each individual additive fed at 0, 4, and 7 g/day, while being housed in a shade-covered, open-sided, naturally ventilated pens. Dry matter intake was 1.02 ± 0.05 kg/day across three experiments (Co, Eo, and EOC), but individual dry matter intakes (DMIs) were not recorded. Thao et al. (22) reported no EO impacts on DMI. For the CARR experiment, three rams with permanent rumen cannula, using the same surgical procedures as described above, weighing ∼50 kg, were arranged in a 3 × 3 Latin square following the same experimental protocol.

Sampling

Ensiled corn silage was sampled weekly and divided into three subsamples: the first subsample was used for DM concentration; the second subsample (100 g) was immediately taken to the laboratory and blended (QE, Zhejiang, China) for ∼60–80 s to be used for measuring ruminal nutrient digestion after weighing into nylon bags (*in situ*); and the third subsample was stored at −20°C for later nutrient analyses. The average corn silage nutrient concentrations across all experiments was DM of 93.0 ± 0.20%, CP of 5.66 ± 0.10%, NDF of 56.0 ± 0.83%, and ADF of 28.2 ± 2.07% on a DM basis.

Ruminal Digestion of Corn Silage, Leaf, and Husk Tissue

At the beginning of each sampling period, three individual nylon bags (400 mesh, 6.5 × 9.5 cm tied with nylon cord) containing 4 g of thoroughly ground fresh corn silage sample were inserted through the ruminal fistula to measure 48-h nutrient digestion. Each ram received three individually labeled rumen nylon bags in each of the three time periods for each additive treatment amounts (0, 4, and 7 g/day) and one empty bag for correction of residual contamination. At the same specified time of nylon bag insertion for 48-h nutrient digestibility, the treatment feeding rate of 0, 4, or 7 g/day of the specific experimental additive (Co, EO, or EOC) were weighed and wrapped in paper in advance and then daily placed directly through the rumen fistula into different ruminal areas to ensure that the specific additive was completely consumed. Thus, consistent with a replicated 3 × 3 Latin square, each ram in each square received each additive amount in the three different Latin square periods. Thus, each treatment additive and its requisite feeding amount resulted in three rams/treatment for a specific time period (i.e., 1, 2, or 3). After 48 h fermentation, all nylon bags attached via a nylon cord were removed and washed gently with 39°C running water until the rinse water was clear. Rinsed bags were then placed in a 65°C drying oven and dried to a constant weight. This nutrient digestibility procedure was performed separately for each of the individual additives (Co, EO, EOC, and CARR) across the four experiments.

Additionally, leaf and husk samples were sourced from the ensiled corn silage that were of sufficient size for cutting into 1 × 1-cm lots. Leaf and husks were selected not only due to their high fiber content but also due to their thin fiber structure for light to illuminate for microscopic scanning. One leaf and husk sample were placed in a separate nylon bag, sealed, and placed through the ruminal fistula into the rumen at the same time as the corn silage nutrient digestibility samples. After 48 h of ruminal digestion and fermentation, the undigested and digested corn silage tissues (leaf and husk) were removed from the rumen, then removed from the nylon bags and placed and fixed to a glass slide containing precooling 2.5% glutaraldehyde solution, then cut into the size of 0.5-cm^2 area with scissors. These leaf and husk corn silage samples were treated using the same procedures

and then prepared for SEM and SM via the procedures of Jiao et al. (23).

Rumen Fluid Collection

After removal of the nylon bags, containing corn silage and leaf and husk, from the rumen at 48 h, subsequently 50 ml of rumen fluid was extracted from each ram during each period and filtered through four layers of cheese cloth into a clean sampling container. The rumen fluid pH was immediately recorded using a pH meter (HI98103, Shanghai, China) with a glass electrode. The rumen fluid was then preserved by freezing in a cryopreservation fridge (−20°C). Samples were thawed and analyzed for NH_3-N (colorimetric method described below) and VFA concentrations.

Laboratory Analysis

All feed and *in situ* residue samples were dried at 65°C (AOAC, 2019; 930.15) in a forced-air oven (DHG - 9240A, Shanghai, China) for 6–8 h to a constant weight and ground through a 1-mm screen and analyzed for CP (990.03), ADF (973.18), and NDF (2002.04) using Association of Official Agricultural Chemists (AOAC) International standard laboratory procedures (2016).

After 48 h of rumen fermentation, the nylon bags containing both corn silage and corn silage leaf and husk were removed and washed with distilled water until the surfaces of the tissues were cleaned. Nutrient analyses for nutrient digestibility were stated previously following the standard methods published by AOAC International (24).

Ruminal VFA Analyses

Ruminal VFA concentrations were measured via high-performance liquid chromatography (Agilent 1100, Santa Clara, CA) to determine formic acid, acetic acid, propionic acid, butyric acid, and lactic acid concentrations following the procedures and chromatographic conditions published by Bai et al. (25).

Rumen NH_3-N Analysis

Ruminal NH_3-N concentrations were measured following the colorimetric methods as described by Feng and Gao (26) using a 721-type spectrophotometer (Zhengzhou Mingyi Instrument Equipment Co., Ltd., Henan, China). The ruminal NH_3-N concentration was calculated from the standard curve having an $R^2 = 0.9988$ (A = 0.3815x – 0.0084), where A is the absorbance, and x is NH_3-N concentration at 700 nm. The 2-ml rumen fluid sample was diluted with distilled water 5-fold prior to chemical reaction.

Scanning Electron Microscopy for Silage Tissue

After 48 h of ruminal fermentation and digestion, the nylon bags containing the corn silage tissue samples of leaves and husk were removed, bags opened and residue collected, followed by washing with distilled water repeatedly until the tissue surface was cleaned. Then, the whole sample of the leaf or husk was placed under an SM (Discovery. V 20, Jena, Germany) at 12.6 × magnification for viewing the fiber structure and any cell contents remaining. After completing the observations under the stereomicroscope, samples were subsequently fixed, rinsed, and dehydrated via freeze drying (freeze dryer VFD-21S, Yamato Scientific Co., Ltd., Koto-Ku, Japan) after which a film coating was applied (Sputter coater, MSP-1S, Hitachi High-Technologies, Minto-Ku, Japan). The prepared leaf and husk samples were viewed using an SEM (S 3400 N, Hitachi Science and Technology, Minto-Ku, Japan) to visually observe and record sample cell fiber microstructure at 50× magnification (23) with photographs taken of each sample from each treatment period for each additive.

Calculation and Statistical Analyses

The calculation of corn silage ruminal nutrient digestibility (DMD, CPD, NDFD, and ADFD) via *in situ* nylon bag was as follows: nutrient digestibility (%) = 100 × (sample nutrient concentration – residue nutrient concentration)/sample nutrient concentration. Before any statistical analyses were conducted, all data were checked for normality and outliers using the univariate procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). The box and whisker plots and Shapiro–Wilk test were used to verify that data were normality distributed ($P > 0.15$). All data for the three treatment additives (Co, EO, and EOC) were subjected to least squares ANOVA for a replicated 3 × 3 Latin square design (27) using the PROC MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) having three inclusion rates of 0, 4, and 7 g/day. The statistical model used was

$$Y_{ijkl} = \mu + I_i + P_j + R(S_1) + S_1 + (T \times S) + e_{ijkl} \quad (1)$$

where Y_{ijlk} = dependent variable, μ = overall mean, I_i = additive inclusion rate, P_j = period, $R(S_1)$ = ram within square, S_1 = square, (I × S) = inclusion rate by square, and e_{ijkl} = experimental error. If the probability of the interaction of I × S was >0.1, this interaction was eliminated from the model. All sources of variation in the statistical model were considered fixed, except for ram within square, which was considered random.

For the CARR experiment, the statistical model used was:

$$Y_{ijk} = \mu + I_i + P_j + R_k + e_{ijk} \quad (2)$$

where Y_{ijlk} = dependent variable, μ = overall mean, I_i = carrier inclusion rate, P_j = period, R_k = ram, and e_{ijkl} = experimental error. All sources of variation were considered fixed, except for ram, which was considered random. Significant differences were declared when $P < 0.05$ and trends declared at $0.05 \leq P < 0.10$.

RESULTS AND DISCUSSION

Carrier Impact on Nutrient Digestibilities

Miscommunication/misunderstanding among the coauthors resulted in the need to conduct a later separate experiment evaluating CARR impact on nutrient digestibility and ruminal fermentation. No differences ($P > 0.10$) were observed in nutrient digestibility and ruminal fermentation when rams were fed CARR at 0, 4, and 7 g/day (**Table 2**), except ruminal NH_3-N concentrations. Rams fed the CARR at 4 g/day demonstrated a lower ruminal NH_3-N concentration compared with rams fed

TABLE 2 | Effect of adding carrier (CARR) on ruminal nutrient digestibility and ruminal fermentation.

Measurement	CARR-0 g	CARR-4 g	CARR-7 g	SEM	$P<^1$
Digestibility					
DM	57.5	57.9	56.6	1.45	0.79
CP	71.1	70.3	68.4	1.88	0.60
NDF	27.1	28.8	26.7	2.80	0.82
ADF	14.4	15.0	15.1	2.97	0.97
Ruminal parameters					
pH	6.07	6.20	6.03	0.32	0.90
NH_3-N, mg/dl	6.1[a]	4.4[b]	5.8[a]	0.38	0.01
Total VFA, mmol/L	95.1	98.6	99.8	19.8	0.99
Acetate, molar %	51.5	52.7	53.0	3.15	0.91
Propionate, molar %	26.6	27.0	26.0	2.84	0.91
Isobutyrate, molar %	1.88	1.34	1.55	0.68	0.67
Butyrate, molar %	12.6	13.3	13.4	4.55	0.98
Isovalerate, molar %	3.28	2.86	2.78	0.65	0.94
Valerate, molar %	3.76	3.10	3.27	0.74	0.74

[1] *Probably of F-test for treatment.*
[a,b] *Means in the same row with differing superscripts differ, P < 0.05.*

TABLE 3 | Effect of adding Co on 48-h ruminal nutrient digestibility of corn silage (%).

Digestibility	Co-0 g	Co-4 g	Co-7 g	SEM	$P<^1$
DM	59.4[b]	63.9[a]	62.4[a]	2.07	0.01
CP	46.4[b]	49.9[b]	57.8[a]	2.51	0.01
NDF	59.4[b]	63.9[a]	62.4[a]	2.07	0.01
ADF	36.3	43.3	31.7	6.50	0.41
Hemicellulose	56.2[b]	63.6[a]	65.9[a]	1.48	0.01

[1] *Probably of F-test for treatment.*
[a,b] *Means in the same row with differing superscripts differ, P < 0.05.*

CARR at 0 and 7 g/day. Even though ingredients in the CARR could be hypothesized to influence ruminal fermentation and nutrient digestibility, the ingredient inclusion rates and feeding rates of the CARR are insufficient to elicit any beneficial response. These data support the conclusion that the CARR is having little to no impact on ruminal fermentation and nutrient digestibility in the experiments reported below. Benchaar et al. (28) reported that only very high EO doses have demonstrated a response (if observed) in animal performance, digestibility, and ruminal fermentation. Therefore, the conclusions in the following main experiment(s) would be considered valid.

Corn Silage Nutrient Digestibilities

Co Feeding Rate

The corn silage ruminal DMD by rams fed 4 and 7 g/day Co was greater ($P < 0.05$) compared with rams fed 0 g/day Co (**Table 3**). The DMD was increased by 4.51 and 3.01%, respectively, compared with rams fed the control (0 g/day). Increasing the Co feeding rate from 4 to 7 g/day did not ($P > 0.10$) further enhance DMD; thus, sufficient DMD enhancement was achieved by feeding 4 g/day Co. The CPD was 11.4% greater ($P < 0.05$) for rams fed 7 g/day compared with rams fed Co at 0 g/day, while rams fed Co at 4 g/day were 7.8% greater but statistically similar ($P > 0.10$) to rams fed 0 g/day. Measured fiber digestion as NDF and/or HC digestibility was greater ($P < 0.05$) for rams fed 4 and 7 g/day of Co compared with rams fed 0 g/day, while ADF digestion was similar ($P > 0.10$) among treatments. Feeding Co at 4 and 7 g/day enhanced NDF and HC digestion by 11.4 and 7.8%, respectively. The HC fiber fraction would be the more easily digestible fiber fraction compared to the cellulose fraction. These results demonstrate that supplementing Co, as soluble Co lactate (vs. cobalt carbonate), improved the ruminal digestion of DM, CP, NDF, and HC, with a 4 g/day feeding rate appearing to be sufficient for improved digestibility, unless greater CP digestion is warranted, which could be accomplished by increasing the feeding rate to 7 g/day feeding rate (**Table 3**).

Previous studies have reported that Co had either significant or numerical improvements for fiber digestion (4, 13, 29). Adding Co to the ration could improve feed digestibility, especially when feeding poor-quality roughages (30). Jiang (31) reported that lactating Holstein cows supplemented with 0.3 mg/kg Co demonstrated improved ($P < 0.05$) cellulose digestibility compared with control. Our results demonstrate that adding 4 g Co lactate resulted in increased NDF and ADF digestibilities of corn silage of 4.5 and 7.01%, respectively, compared with the control (0 g/day) fed rams. These results may be explained by some microbial communities needing more Co and/or because Co forms a crosslink between negatively charged bacteria and negatively charged feeds (32). Zelenak et al. (33) showed that the supplementary feeding of Co to goats significantly increased hay digestibility. Feeding Co also improved corn silage DMD and CPD in this study, which was similar to results in the cattle (34), where authors reported that Co improved cattle BW gains when feeding non-leguminous hay combined with urea improved cellulose digestion. Hatfield (35) cited work that proteins are frequently associated with lignin–carbohydrates complexes, and the speculation is that enhance fiber digestion results in greater CP digestion.

In our study, NDFD and HCD increased with 4 g of Co, while ADFD was similar among all Co inclusion rates, demonstrating that supplementing 4 g was optimal for fiber degradation. These results are similar to the results reported by Liu et al. (36) in rabbits demonstrating that daily gain and feed efficiency were improved with a moderate Co inclusion rate, but a larger Co inclusion rate resulted in poorer BW gain and feed efficiency. Other studies confirmed that a specific Co amount in the ration can maintain the amount and type of rumen bacteria and parasites at normal levels, but excess Co can hinder their growth (37). Therefore, Co supplementation requires careful control ration inclusion rates. A large number of experimental studies have suggested that supplementing 5 mg/kg Co chloride (soluble like Co lactate) to sheep daily can improve ruminal fiber digestibility (38). Co concentrations above 0.50 mg/kg were appropriate for goats (39), whereas a concentration of 0.25 mg/kg is adequate for sheep (40). In this study, feeding 4 g Co lactate was adequate without being excessive.

TABLE 4 | Effect of adding essential oils (EO) on 48-h ruminal nutrient digestibility of corn silage (%).

Digestibility	EO-0 g	EO-4 g	EO-7 g	SE	$P<$[1]
DM	55.3	56.0	54.9	3.57	0.98
CP	46.5	49.1	44.5	5.42	0.67
NDF	38.0	36.6	36.5	4.75	0.96
ADF	28.5	27.2	30.1	6.22	0.89
Hemicellulose	64.1[b]	71.4[a]	69.1[ab]	1.28	0.01

[1] *Probably of F-test for treatment.*
[a,b] *Means within same row with differing superscripts differ, P < 0.05.*

TABLE 5 | Effect of adding a cobalt and essential combination (EOC) on 48-h ruminal nutrient digestibility of corn silage (%).

Digestibility	EOC-0 g	EOC-4 g	EOC-7 g	SE	$P<$[1]
DM	57.7	60.0	60.0	2.85	0.20
CP	38.3	36.8	37.6	6.01	0.99
NDF	21.4[b]	28.8[a]	27.7[a]	5.02	0.01
ADF	24.3[b]	33.3[a]	34.4[a]	5.74	0.02
Hemicellulose	70.9	73.5	72.6	0.88	0.22

[1] *Probably of F-test for treatment.*
[a,b] *Means within the same row with differing superscripts differ, P < 0.05.*

EO Feeding Rate

Ruminal corn silage DM, CP, and NDF digestibilities were similar ($P > 0.10$) among rams fed all treatments (**Table 4**). Corn silage HC digestibility was 7.3% greater ($P < 0.05$) for rams fed EO at 4 g/day compared with rams fed 0 and 7 g/day. Rams fed 7 g/day of EO was similar ($P > 0.10$) compared with rams fed 0 g/day (control) but numerically improved 5.0%, which indicates that more animals were needed to become significant.

In this study, the corn silage ruminal DM, CP, NDF, and ADF digestibility demonstrated no influences by EO or increasing EO feeding rates but was numerically increased by 0.65 and 2.58%, respectively, when supplemented with 4 g/day EO compared with the 0 g/day EOC (control). Oregano essential oil increased nutrient digestibility and milk protein concentration by dairy cows (41), which is consistent with proposed EO mechanisms. Several EO are known to stimulate appetite, activate digestive enzymes through biofeedback, change chyme viscosity, and increase feed intake (42, 43). However, ruminal nutrient digestibilities were similar with increasing EO feeding rates in this study using sheep, which could potentially be explained by the test animal species, EO feeding rate, and/or different metabolic mechanisms (i.e., ruminal vs. postruminal) by different species. Thao et al. (22) reported similar nutrient (DM, CP, NDF, and ADF) digestibilities when feeding Eucalyptus oil to water buffalos. Froehlich et al. (7) suggested that in the past, EO may have been fed at too high of a feeding rate, thereby preventing performance improvements.

EOC Feeding Rate

The corn silage ruminal NDF and ADF digestibilities were greater ($P < 0.05$) for rams fed the EOC combination at 4 and 7 g/day compared with rams fed 0 g/day (control; **Table 5**). The DM, CP, and HC digestibilities were similar ($P > 0.10$) among rams receiving all treatments. Feeding the EOC combination above 4 g/day resulted in little improvement in ruminal nutrient digestibility.

Studies have shown that EOC is beneficial to ruminants consuming high-fiber diets. The results indicated that the fiber digestibility increased by 12–22% when using EOC at Oklahoma State University (unpublished). Kuester (44) found that the roughage digestibility of DM, CP, NDF, and ADF were improved by 2.3, 3.5, 3.8, and 4.2%, respectively, by lactating dairy cows fed the same EOC product at 28 g/day, compared with lactating cows fed 0 g/day EOC (control), similar to this experimental conclusion, where the DMD improved by 2.29 and 2.33% when sheep were fed 4 and 7 g EOC. The NDFD and ADFD improved by 6.30–7.39 and 9.05–10.12%, respectively ($P < 0.05$). As noted above, oregano EO are natural feed additives. Oregano EO can alter the intestinal microbial community structure and optimize the animal gut microflora environment (13). Cobalt is one of the indispensable trace elements and plays a crucial role in crude fiber digestion. Both are present in EOC and play coordinated roles to improve the rumen environment, enhancing the activity of cellulolytic bacteria to promote the rumen fermentation functions and forage utilization. For CPD, NDFD, ADFD, and HCD, there were no significant differences between 4 and 7 g ($P > 0.05$), which suggested that a 4-g EOC inclusion rate is adequate for sheep.

Based on the current evaluation of different additives, we found that adding EO alone could enhance ruminal HC digestion, which would be in agreement with Thao et al. (22). Thao et al. (22) reported improved NDF digestibility with similar ADF digestibility, which indicates that HC digestibility was improved with EO. Co significantly improved the corn silage ruminal DM, NDF, and HC digestibility, illustrating that Co in combination with EOC probably played an important role in straw fiber digestion and degradation; however, EO may still be involved in the maintenance of the rumen microbial flora. There is at least a numerical indication of synergism between Co and EO (oregano). If it is assumed that the oregano EO alone has a small negative influence on NDFD and ADFD (−1.4 and −1.3%, respectively) and Co alone improved NDFD and ADFD by 4.5 and 7.0%, respectively, then it could be expected that the combination would result in improvements in NDFD and ADFD of 3.1 and 5.7%, respectively, assuming no synergism or antagonism. However, supplementation of the EO and Co combination (EOC) resulted in NDFD and ADFD improvements of 7.4 and 9.1%, respectively. While not definitive, this does suggest the need for further research into mechanisms involved in the activities of oregano EO and Co and their possible synergism related to ruminal fiber digestion by ruminants.

Ruminal Fermentation

Co Feeding Rate

Ruminal pH was similar ($P > 0.10$) for rams fed all Co inclusion rates (**Table 6**). In contrast, rams fed Co at 7 g/day

TABLE 6 | Effect of Co feeding rate on sheep ruminal characteristics.

Measurement	Feeding rate of Co, g/day			SEM	$P<^1$
	0	4	7		
pH	6.37	6.34	6.16	0.07	0.20
NH_3-N, mg/dl	9.99[b]	11.1[b]	15.8[a]	0.72	0.01
VFA, mmol/L					
Total	62.2[b]	59.4[b]	71.7[a]	0.75	0.01
Acetate	47.7[b]	47.7[b]	55.3[a]	1.24	0.01
Propionate	12.0	11.7	11.3	0.46	0.61
Butyrate	5.14	5.15	5.20	0.10	0.39
Acetate/propionate ratio					
	3.98	4.07	4.89	0.40	0.11

[1] *Probably of F-test for treatment.*
[a,b] *Means within the same row with different superscripts differ, P < 0.05.*

TABLE 7 | Effect of essential oils (EOs) feeding rate on sheep ruminal characteristics.

Measurement	Feeding rate of EO, g/day			SEM	$P<^1$
	0	4	7		
pH	6.35[b]	6.29[b]	6.53[a]	0.04	0.03
NH_3-N, mg/dl	14.2	14.0	11.3	0.91	0.15
VFA, mmol/L					
Total	53.1	64.7	65.7	4.03	0.16
Acetate	48.1	49.2	49.1	1.24	0.65
Propionate	14.4	14.7	15.0	2.23	0.16
Butyrate	5.07	5.43	4.95	0.80	0.65
Acetate/propionate ratio					
	3.44	3.60	3.28	0.88	0.91

[1] *Probably of F-test for treatment.*
[a,b] *Means within the same row with different superscripts differ, P < 0.05.*

demonstrated greater ($P < 0.05$) ruminal NH3–N, total VFA, and acetate concentrations compared with rams fed 0 and 4 g Co/day. Concentrations of propionate, butyrate, and the acetate to propionate ratio were similar ($P > 0.10$) among rams fed all Co inclusion rates. The CPD increase observed in **Table 3** may probably be associated with the increase in ruminal NH3–N concentrations, while the increases in DM, NDF, and HC digestibilities may be influencing increases in total VFA and acetate concentrations. Greater fiber digestion would be expected to increase ruminal acetate concentrations.

EO Feeding Rate

Rams fed EO at 7 g/day had greater ($P < 0.05$) ruminal pH compared with rams fed 0 and 4 g/day (**Table 7**). Ruminal NH3–N, total, and individual VFA concentrations were similar ($P > 0.10$) among rams fed all EO inclusion rates. Even though increasing EO inclusion rate increased HC digestibility (**Table 4**), the increased HC digestibility demonstrated little influence on ruminal fermentation parameters, except pH (**Table 7**). In agreement, Thao et al. (22) reported Eucalyptus having no effect on ruminal pH and NH3–N concentrations.

EOC Feeding Rate

Feeding 4 and 7 g/day of the EOC combination resulted in rams having a lower ($P < 0.05$) ruminal pH than rams fed EOC at 0 g/day (**Table 8**). Feeding a combination of Co and EO (EOC) influences ruminal pH in contrast to feeding the individual Co and EO components. Ruminal NH3–N concentrations were similar ($P > 0.10$) among rams fed all inclusion rates, while total VFA concentrations were lower ($P < 0.05$) for rams fed EOC at 7 g/day compared with rams fed 0 and 4 g/day. Rams fed EOC at 4 g/day had greater acetate concentrations than rams fed 0 g/day, while rams fed EOC 7 g/day had the lowest ($P < 0.05$) acetate concentrations compared with rams fed EOC at 0 and 4 g/day. No differences ($P > 0.10$) were observed for propionate, butyrate, and acetate to propionate ratios.

Rumen fluid pH can be a direct ruminal fermentation rate indicator, which usually changes rapidly with feeding time and feed types, ranging from ∼5.0 to 7.5 (45). In this experiment,

TABLE 8 | Effect of essential oils and cobalt combination (EOC) feeding rate on ruminal characteristics of sheep.

Measurement	Feeding rate of EOC, g/day			SEM	$P<^1$
	0	4	7		
pH	6.00[a]	5.82[b]	5.77[b]	0.04	0.01
NH_3-N, mg/dl	15.4[a]	15.0[a]	14.4[b]	0.41	0.29
VFA, mmol/L					
Total	64.8[a]	64.9[a]	60.9[b]	1.28	0.04
Acetate	52.4[b]	54.0[a]	48.5[c]	0.15	0.01
Propionate	10.9	10.9	10.6	0.14	0.13
Butyrate	4.71	4.96	5.33	0.41	0.62
Acetate/propionate ratio					
	4.83	4.96	4.58	1.08	0.22

[1] *Probably of F-test for treatment.*
[a,b,c] *Means within the same row with different superscripts differ, P < 0.05.*

ruminal pH was lowered when rams were fed the EOC combination compared to rams fed the 0 g/day EOC (control; $P < 0.05$). However, ruminal pH of all additives evaluated in this study at different inclusion rates were within normal ranges, indicating a normal functioning ruminal environment. These results suggest that feeding the EOC combination could be implemented compared with Co or EO independently to effectively reduce ruminal pH, thereby increasing bacteriostatic efficacy without incurring acidosis.

Wang (46) found that adding 0.1 mg/kg of Co increased artificial rumen pH by 0.32%, but pH of the Co treatment was similar to the control ($P > 0.05$). With the addition of 1.0 mg/kg of Co, the pH rose to 6.31, greater than the control by 0.96% ($P < 0.05$), which demonstrated that ruminal pH rose with increased Co. However, Miao (47) found that pH had a tendency to decrease with the addition of different levels of Co in an *in vitro* experiment using an artificial rumen ($P > 0.05$), which is similar to the present results. In summary, regardless of Co inclusion rate, the average ruminal pH was in the normal range,

which was advantageous to fiber digestion, in addition to nutrient fermentation and rumen microbial growth.

The NH_3-N concentration can reflect the feed N content, N solubility, and degradation rate along with intake of these components. Generally, the ruminal NH_3-N concentration range is 10–50 mg/dl. This value will usually peak ∼1.5–1 h after feeding (48). The NH_3-N concentration changed in the range of 9.99–15.8 mg/100 ml under the three additives and their inclusion rates, indicating that each feed additive did not alter rumen NH_3-N beyond normal limits. The NH_3-N concentrations increased with increasing Co inclusion rate that was different from increasing EOC and EO inclusion rates. Co is a vitamin B_{12} component, which plays an important role in animal protein metabolism (40). The Co may have provided positive ruminal fermentation regulation by being beneficial to rumen microbial activity (47, 48). Feeding EO and EOC numerically reduced NH_3-N concentrations, but these differences were not significant ($P > 0.05$), which may be due to EO promoting the growth and proliferation of rumen microbes (40, 49). Thymol in EO will act on rumen microorganisms and affect amino acid deamination (50, 51). All of these results indicated that EO and EOC could potentially impact ruminal NH_3-N concentrations, but Thao et al. (22) reported no impact on NH_3-N concentrations.

Ruminal VFA concentrations reflect microbial activity, ruminal absorption, and/or ruminal passage rates (48). Increasing Co inclusion rates influenced TVFA and acetic acid ($P < 0.05$) but demonstrated little impact on propionic acid ($P > 0.05$) concentrations. The TVFA and acetic acid concentrations were greater for rams fed 7 g/day than rams fed 0 (control) and 4 g/day inclusion rates ($P < 0.05$). This difference is likely due to Co being a critical trace mineral for rumen microbial activity. Some microbes can use Co to synthesize vitamin B_{12} and other rumen microbial growth factors (52). Vitamin B_{12} synthesis is necessary for many bacterial enzymes, and these enzymes participate propionic acid metabolism along with synthesis of methane, acetic acid, and methionine (3, 53). Research using diets comprised mainly of wheat straw demonstrated that increasing Co inclusion rates significantly increase TVFA and acetic acid, propionic acid, and butyric acid production ($P < 0.05$; 27). However, Hussein et al. (29) and Tiffany et al. (54) concluded that there was no effect on fermented liquid TVFA using *in vitro* culture condition, which may be related to the animal species, test diet, trial duration, and/or other factors. In contrast, Thao et al. (22) reported that Eucalyptus oil decreased ruminal acetate and increases ruminal propionate concentrations in water buffalos, which may indicate that varying the EO will have varying impacts on ruminal fermentation.

In general, increasing Co inclusion rates increased TVFA and acetic acid concentrations, while EO (oregano) demonstrated minimal impacts for VFA, and finally, the EOC combination decreased TVFA and acetic acid at increasing inclusion rates, i.e., 7 g/day. It could be possible for a synergistic effect between Co and EO (EOC combination) improved rumen wall VFA absorption to maintain normal ruminal metabolic function, causing VFA concentrations to decline. This speculation needs to be evaluated in future research.

SEM Scans of Corn Silage Husk and Leaf Fiber Structures After Ruminal Digestion

The corn silage husks and leaves were incubated in the rams using nylon bags for ruminal *in situ* degradation. Each figure (**Figures 1–8**) contains micrographs of a sample group comprised of a preruminal digestion scan of the raw material and then scanned after 48 h of ruminal *in situ* degradation with increasing additive inclusion rate. These scans were visually appraised because, to our knowledge, no mechanism exists, nor could we find or develop one, to convert these scans into a measurement (absorbance, area, volume, etc.) that could be statistically evaluated. Therefore, the SEM and SM scans on plant cell structure were visually evaluated and used to confirm the measured digestibility parameters presented in the preceding tables. In addition, the ruminal *in situ* samples for each additive (Co, EO, and EOC) from each sampling period were visually appraised and found to be fairly consistent (minimal visual variation) across each ram for each period. Thus, the figures containing the scans are presented as being representative of the entire additive across periods at the increasing inclusion rates for that experiment.

Co Feeding Rate

The corn silage husk samples visually demonstrated dense epidermal trichomes and integrated skin before ruminal digestion (**Figure 1A**), while increasing Co inclusion rate visually demonstrated increasing surface area digestion by ruminal microbes when rams were fed Co at 4 and 7 g/day compared with rams fed Co at 0 g/day (**Figure 1D**). For rams fed increasing Co inclusion rates at 4 and 7 g/day, the trichomes and mesophyll were completely digested leaving only disarrayed parallel veins compared to rams fed Co at 0 g/day, which demonstrated only partial digestion.

The corn silage leaf ruminally incubated *in situ* visually demonstrated a neatly arranged epidermal cells with wax, epidermal trichomes, guard cells, closed stomas, whole mesophyll, and vein structure (**Figure 2A**). Compared with the ruminally undigested material, feeding Co at 0 g/day demonstrated visual disappearance of epidermal trichomes and broken surfaces with partially digested mesophyll. However, by increasing Co inclusion rates to 4 and 7 g/day fed to the rams, the ruminal degradation demonstrated a visually complete digestion of leaf epidermal cells, palisade tissue, and spongy tissue leaving only the netted veins (**Figures 2B–D**).

EO Feeding Rate

Prior to ruminal *in situ* degradation, the corn silage husks cells were neatly arranged, with clear cell walls, completed cell frames, and visible epidermal trichomes with veiny and hairy stomas (**Figure 3A**). Compared with the preruminal degradation husk, rams fed all EO inclusion rates demonstrated that the corn silage husk was completely digested, leaving only the epidermal cells, palisade tissue, or spongy tissue, with smooth and neatly arrayed parallel veins (**Figures 3B–D**).

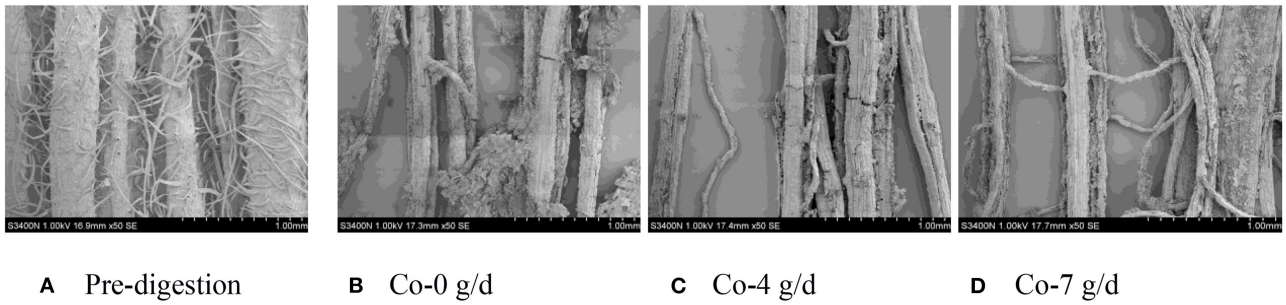

FIGURE 1 | (A–D) Scanning electron microscope (SEM) images at 50× magnification demonstrating corn silage husk degradation and remaining fiber structure when rams were fed cobalt at 0 (Co-0), 4 (Co-4), or 7 g/day (Co-7).

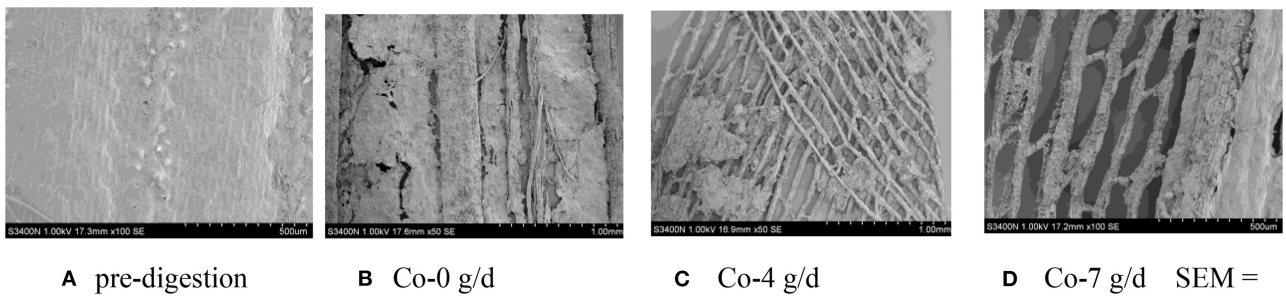

FIGURE 2 | (A–D) Scanning electron microscope (SEM) images at 50× magnification demonstrating corn silage leaf degradation and remaining fiber structure when rams were fed cobalt at 0 (Co-0), 4 (Co-4), or 7 g/day (Co-7).

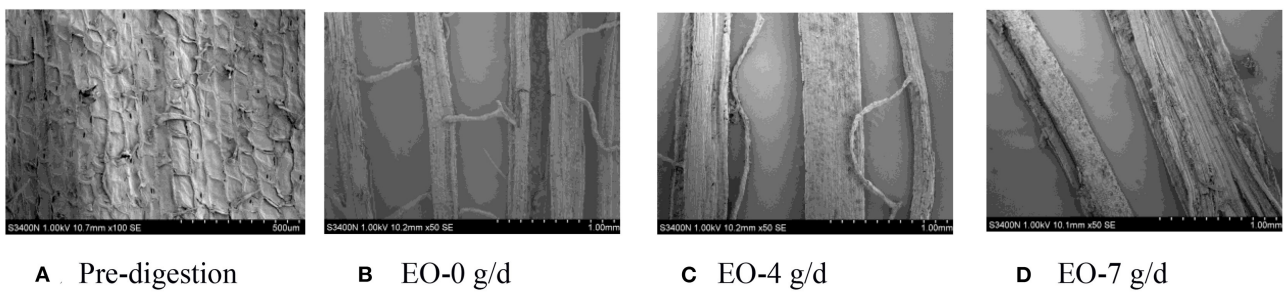

FIGURE 3 | (A–D) Scanning electron microscope (SEM) images at 50× magnification demonstrating corn silage husk degradation and remaining fiber structure when rams were fed essential oils (EOs) at 0 (EO-0), 4 (EO-4), or 7 g/day (EO-7).

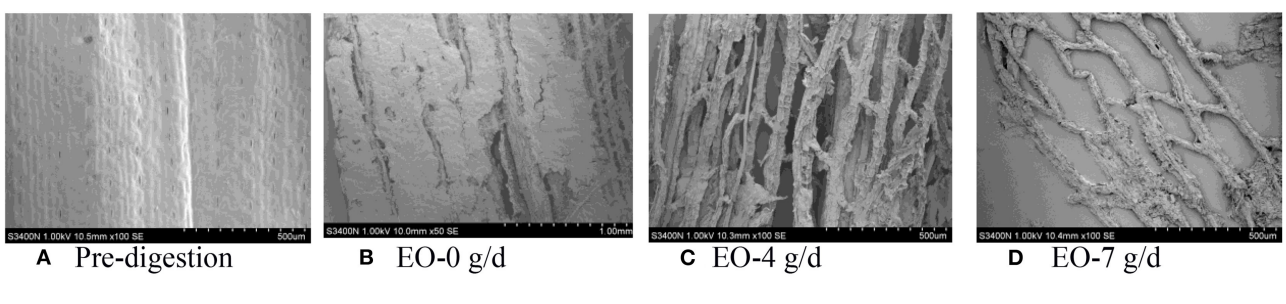

FIGURE 4 | (A–D) Scanning electron microscope (SEM) images at 50× magnification demonstrating corn silage leaf degradation and remaining fiber structure when rams were fed essential oils (EOs) at 0 (EO-0), 4 (EO-4), or 7 g/day (EO-7).

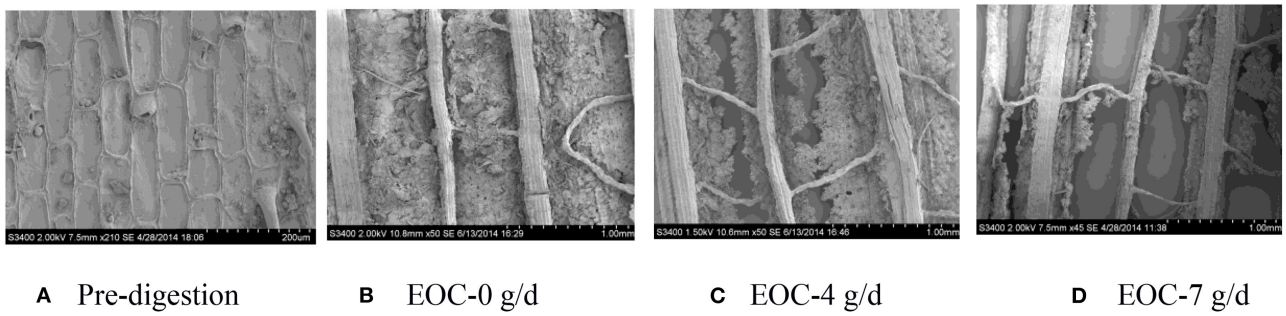

FIGURE 5 | (A–D) Scanning electron microscope (SEM) images at 50× magnification demonstrating corn silage husk degradation and remaining fiber structure when rams were fed cobalt and essential oils (EOCs) at 0 (EOC-0), 4 (EOC-4), or 7 g/day (EOC-7).

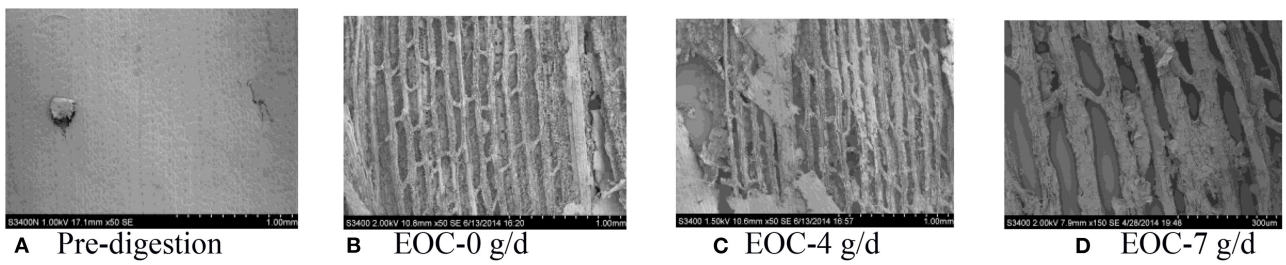

FIGURE 6 | (A–D) Scanning electron microscope (SEM) images at 50× magnification demonstrating corn silage leaf degradation and remaining fiber structure when rams were fed cobalt and essential oils (EOCs) at 0 (EOC-0), 4 (EOC-4), or 7 g/day (EOC-7).

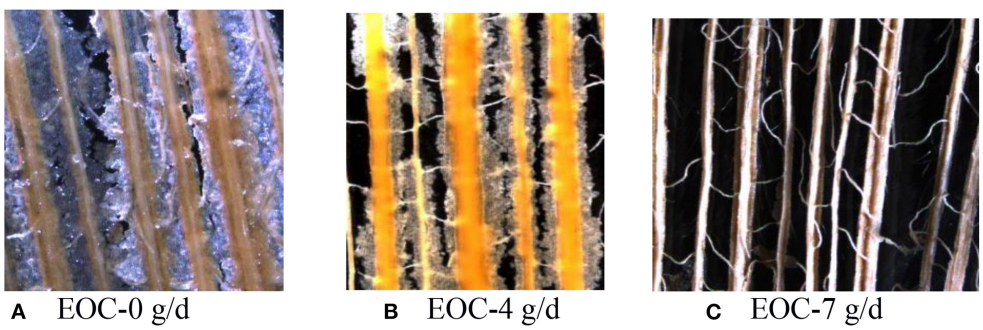

FIGURE 7 | (A–C) Stereo microscopy (SM) scans at 12.6× magnification demonstrating corn silage husk degradation and remaining fiber structure when rams were fed cobalt and essentials (EOCs) fed at 0 (EOC-0), 4 (EOC-4), or 7 g/day (EOC-7).

Similar to the corn silage husks, the preruminal *in situ* degradation of the corn silage leaf epidermal cells were neatly arranged with clear cell walls and complete cell frames, along with visible epidermal trichomes and veiny and hairy stomas (**Figure 4A**). Compared to the preruminal degradation, rams fed 0 g/d EO demonstrated that the plant cell wax layer was damaged, leaving the palisade and spongy tissues outside; the mesophyll was partially destroyed, with complete veins, but no epidermal trichomes (**Figure 4B**). Rams fed 4 g/day EO demonstrated that mesophyll was partially degraded after 48 h (**Figure 4C**) compared to rams fed EO at 0 g/day, while the rams fed 7 g/d EO demonstrated that the mesophyll was completely degraded with only some thin and clear reticulate veins remaining (**Figure 4D**).

EOC Feeding Rate

The preruminal *in sit*u degradation of the corn silage husk was as described previously (**Figure 5A**). Feeding rams 0 g/day EOC (the combination of Co and EO) demonstrated damaged husk epidermal wax and mesophyll with a clear cell wall (**Figure 5B**) compared to the preruminal sample. Compared to rams fed 0 g/d EOC, feeding 4 g/day EOC demonstrated a partially digested palisade tissue and mesophyll (**Figure 5C**), compared with feeding 7 g/day EOC, which demonstrated a more complete husk degradation with only veins remaining visual (**Figure 5D**).

The preruminal *in situ* degradation of the corn silage leaf material was as described previously (**Figure 6A**). Feeding rams 0 g/day EOC after 48 h of ruminal degradation demonstrated a

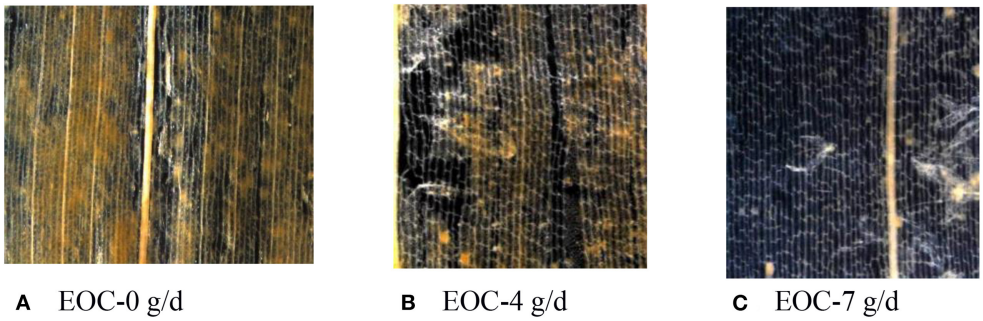

FIGURE 8 | (A–C) Stereo microscopy (SM) scans at 12.6× magnification demonstrating corn silage leaf degradation and remaining fiber structure when rams were fed cobalt and essentials (EOCs) fed at 0 (EOC-0), 4 (EOC-4), or 7 g/day (EOC-7).

visually damaged wax layer with some epidermal cell degradation compared with the preruminal sample (**Figure 6B**). Compared to rams fed 0 g/day EOC, rams fed 4 g/day EOC demonstrated the leaf epidermal cells being completely digested with leaving only spongy tissue outside, while degrading part of the mesophyll (**Figure 6C**). Rams fed 7 g/day EOC demonstrated a complete leaf degradation compared with rams fed lower EOC inclusion rates (**Figure 6D**).

Stereomicroscope Scans After Ruminal Digestion

The effort required to process samples for SM scanning along with our major interest in the EOC combination performance resulted in selecting only samples from rams fed increasing EOC inclusion rates for SM scanning at 12.5× magnification. Feeding rams increasing EOC inclusion rates visually demonstrated increasing corn silage husk degradation (**Figures 7A–C**), with rams fed 7 g/day EOC demonstrating remaining husks containing only clear mesh veins. Corn silage leaves demonstrated the same increasing degradation with increasing EOC inclusion rates with rams fed 7 g/day EOC in which the leaf residue was completely transparent with only the vein structure remaining (**Figures 8A–C**).

Previous Co and EO studies have focused on the feeding responses as measured by animal production performance, nutrient utilization, and livestock immune function (7, 17, 44). To our knowledge, there are no scientific literature studies, and this is the first time that SEM and SM scans were used to study sheep ruminal straw cell fiber degradation in the absence or presence of different feed additives. While quantification would be preferred, future studies may be able to develop procedures to quantify ruminal digestion using SEM and SM techniques. However, in this study, SEM and SM techniques provided a direct visual demonstration to supplement and compliment ruminal digestibility measurements for the first time.

Plant cell walls and fibrous components of cellulose, hemicellulose, and lignin are usually the most difficult material for ruminant animals to degraded (55). Straw plant surfaces are covered with a wax layer and cuticle, which is difficult to be rapidly degraded by ruminal microbes and enzymatic degradation. Ruminal microbes depend mainly on a damaged surface and stoma to initiate degradation and digestion. Therefore, cells located in the inner layer are digested later because the plant cells are tightly arranged with overlapping cell walls (56). Thus, rupturing the plant cell wall will expose cell contents, which is critical for feed degradation, nutrient digestion, and feed additive effectiveness.

In this study, when sheep were fed 4 and 7 g/day Co, the epidermal cells, palisade tissue, and spongy tissue of corn silage leaves were completely degraded, with only net-like veins remaining compared with rams fed 0 g/day Co. The corn husk exhibited similar degradation changes under the same conditions with the leaf mesophyll between veins disappearing completely with disorganized parallel veins remaining when rams were fed 4 and 7 g/day. These observations support the conclusion that Co is a microbial catalyst that can improve ruminal microbial activity, especially for cellulosic microbes. Improving ruminal microbial cellulosic activity will enhance fiber digestibility of straw (3, 30, 31, 37, 40, 57, 58).

When feeding increasing EO inclusion rates to rams, the ruminal degradation of the corn silage husk and leaf resulted in morphological changes similar to those observed with feeding increasing Co inclusion rates. The speculation is that plant phenolic compounds maintained the ruminal microbial system balance for the combination of ruminal bacteria, protozoa, and fungi for completing plant cell wall degradation (56).

Feeding increasing EOC inclusion rates demonstrated increased ruminal leaf degradation, but an apparent synergistic effect did not occur when combining Co lactate and EO on fiber degradation. The apparent lack of synergism may be due to each additive's independent efficacy to facilitate fiber digestion. Oregano EO is known to exert bacteriostatic efficacy, which may maintain ruminal microbial balance to ensure a healthy rumen with normal fermentation. The soluble Co lactate provides an essential trace element for maintaining gut biome health. With each demonstrating slightly different mechanisms, a combination of Co and EO would ensure greater degradation of straw and improve feed nutrient and fiber digestibility.

CONCLUSIONS

These results suggested that the optimal feeding rate would be 4 g/day of Co or EO, which would be ∼27 g/day for large frame ruminants (i.e., 650 kg). The integration of digestibility estimates with SEM and SM micrographs of digested forage particles can be beneficial in visually confirming nutrient digestibility estimates. Corn silage husk has a high NDF and HC content that is usually very digestible, as visually confirmed by the SEM. Corn silage husk is composed mainly of xylose, which is a highly digestible sugar compared to arabinose. In contrast, corn silage leaves are high in NDF and ADF concentrations, resulting in a high cellulose content, which has much greater variation in the observed digestibility, suggesting that feed additives, such as oregano EO and Co, could enhance cellulose digestibility. The use of SEM and SM may be beneficial when evaluating nutrient digestibility technologies to enhance ruminal nutrient digestion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

TJ was responsible for the trial implementation, supervision of students collecting and analyzing samples, and manuscript preparation. JW is the overall project leader providing financial support and experimental conception. DC was involved in data analyses, statistical analyses, language revisions, journal selection, and manuscript submissions and revisions. DD, MB, and BH contributed to experimental design and providing additives evaluated in the study. SZ contributed to the supervision and assistance of students in managing animals, collecting, and analyzing samples. JL was an undergraduate student assisting with sample analyses, scanning electron microscope, and data collection and organization. ZL contributed to supervision of additive inclusion, sample collection and analysis, and manuscript editing. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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