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The Effect of Alpine Herbs on the Microbiota of In Vitro Rumen Fermentation

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Abstract: Milk from cows grazing on alpine pastures has higher quality than milk from indoor-fed cows, likely due to diet-driven differences in rumen microbiota. We assessed the effects of supplementing alpine herbs—each varying in its content of fiber, protein, and polyphenol—on rumen microbiota via in vitro fermentation, comparing these to a grass hay control using metagenomic sequencing. Fermentations with alpine herbs compared to grass hay control had higher content of fibrolytic *Prevotella* and lower abundances of *Butyrivibrio*, *Ruminococcaceae*, *Anaerovibrio*, *Succiniclasticum*, and *Desulfovibrio*. Fermentations with high starch content (*Alchemilla vulgaris*, *Gallium odoratum* and *Sanguisorba officinalis*) had low, microbial diversity, while fermentations with high content of structural fibre (*Sisymbrium officinale*, *Tanacetum vulgare*, and *Cicerbita alpina*) had high microbial diversity. *C. alpina*, *Sa. officinalis*, and *T. vulgare* fermentations that had high lignin content showed a higher abundance of Bacteroidetes and a lower abundance of Firmicutes. Fermentations with high protein content (*G. odoratum* and *T. vulgare*) induced higher abundance of fibrolytic *Lachnospiraceae*. *Sa. officinalis* and *A. vulgaris* fermentations with high content of polyphenols were associated with increased abundances of *Streptococcus* and family RF-16 and lower abundances of family BS11 and *Desulfovibrio*. Fermentations with *C. alpina* and *Si. Officinale* induced higher abundance of fibrolytic *Fibrobacter succinogenes*. The beta diversity between fermentations corresponded to differences in the contents of protein, lignin, and polyphenols in the plant material. In conclusion, different herbs can promote the abundance of various fibrinolytic bacteria and change the microbial diversity, which has potential to increase the feed efficiency and the robustness of microbiota and reduce methane production.

Keywords: rumen microbiota; alpine herbs; in vitro fermentation; metagenomic sequencing; nuclear magnetic resonance spectrometry

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1. Introduction

The Trentino dairy industry is an important part of the region's economy and culture. The cow herds are stabled indoors; however, dairy farmers regularly move part of their cow

herd to highland farms called *Malga* (>1400 m.a.s.l.) from late spring to early autumn. When moved to highland farms, cows experience a drastic change of habits (i.e., environment, diet, and physical activity when grazing), affecting the quality, microbiota, and composition of the milk and dairy products [1–4]. In general, the dairy cows transferred to summer highland pastures produced a milk increased in lactic acid bacteria taxa, bifidobacteria, and propionibacteria, and reduced in spoilage bacteria [2]. Considering the lipidic profile of the mountain cheeses, total unsaturated fatty acids were significantly higher in the cheeses made from milk from pastured animals than in those made from milk of animals kept indoors, and conjugated linoleic acid isomers again were more represented in the cheeses made with "pasture milk" [3]. The dairy products made using milk from *Malga* and highland pastures are preferred by consumers because of the characteristic taste and smell of the products [5]. These differences are believed to be driven by changes to the cows' diets via modulation of the rumen microbiota [6].

The rumen environment is highly complex and notoriously difficult to predict or control; it is sometimes referred to as a "black box". It contains a microbial population, numbering in the trillions, that is essential for the digestion of plant fibres into volatile fatty acid (VFA) and for the digestion of plant material by the cows [7,8]. It has been estimated that the microbial activity in the rumen produces up to 70% of the energy requirement of the host [9].

A global interspecies core ruminal microbiome has been observed, consisting of *Prevotella*, *Bacteroidales*, *Clostridiales*, *Ruminococcaceae*, *Lachnospiraceae*, *Ruminococcus*, and *Butyrivibrio* [10]. Modulation of the rumen environment can improve feed efficiency [11–13], reduce methane production [14], and alter VFA production in the rumen [15,16]. The rumen microbiota and their VFA production are also directly correlated with the fatty-acid profile of cow milk [17]. Additionally, some species of herbs found in alpine habitats are known for their anti-microbial properties [18]. Therefore, alpine pasture herbs can be expected to modify ruminal fermentation in general, which could also affect methanogenesis. On the level of individual alpine plant species, a limited variation of methanogenesis has been demonstrated in vitro [19]. However, a direct comparison of alpine herbs with lowland/indoor diets in terms of ruminal fermentation is lacking. Previous studies investigating the effects of pastural herbs on the rumen environment have mainly focused on fermentation parameters and gas production. Any study of the microbial aspects has been limited to quantification of total bacteria and/or protozoa [19–21].

In our study we aim to investigate the effects of individual alpine herbs on the cow-rumen microbiota by means of in vitro rumen fermentation. This study attempts to test whether herbs from alpine pastures influence ruminal microbiota differently than those of a lowland/indoor diet, and to indicate how a diet of alpine herbs can modulate methanogenesis. We compared in vitro rumen fermentations (RF) with additions of the alpine herbs most frequently present on alpine pastures in Trentino, namely, *Alchemilla vulgaris* (RF-Alc), *Cicerbita alpina* (RF-Cic), *Galium odoratum* (RF-Gal), *Sanguisorba officinalis* (RF-San), *Sisymbrium officinale* (RF-Sis), and *Tanacetum vulgare* (RF-Tan). These herbs are typical of the Eastern Italian Alps [22] and are known to be rich in bioactive compounds such as tannins and other polyphenols, essential oils, and saponins. These compounds modulate microbial activity, and thereby the rumen environment. Supplementation with *Lolium multiflorum* Lam. grass hay has been used as control batch (CTRL) for the purpose of comparison with a typical plant included in the lowland/indoor diets. In addition, those fermentations were compared to the collected rumen (Rumen) and rumen + medium solution before fermentation (Blank). The chemical data obtained by the same experimental plan are part of a previous work under publication.

2. Materials and Methods

2.1. Ethics Statement

All experiments and procedures were performed according to the Italian animal welfare laws. The obtained cow-rumen material was collected after approval from the Ethical Committee of the University of Padova (Italy). The approval number for these experiments is OPBA 1312041/2022.

2.2. Herb Samples and Composition

The herbs chosen for the experiment belong to herbal varieties found in the alpine pastures of the Vezzena highland (Trento, Italy). Six herbs have been chosen: *Alchemilla vulgaris* L. (Alc), *Sanguisorba officinalis* L. (San), *Tanacetum vulgare* L. (Tan), *Cicerbita alpina* (L.) Wallr. (Cic), *Galium odoratum* (L.) Scop. (Gal), and *Sisymbrium officinale* (L.) Scop (Sis); all were harvested at beginning of the earing stage. Along with these, *Poaceae* grass hay (*Lolium multiflorum* Lam.) was used as the control treatment (CTRL). Our herbal samples were collected at their balsamic period, using leaves and top shoots, while the grass hay CTRL was collected at the beginning of flowering stage.

All of the tested herbs were supplied as dry samples by the Edmund Mach Foundation (FEM, San Michele all' Adige, Trento, Italy). The samples were ground using an ultra-centrifugal mill (Retsch ZM 200, Retsch GmbH, Haan, Germany) with a grinding grid of 1 mm. The ground samples were used for both in vitro fermentation and chemical analysis.

The chemical composition of these herbs is part of a work presently accepted by Massaro et al. [23] The dry matter (DM) content was similar in all supplements (89.7–94.1 g). *A. vulgaris* and *G. odoratum* had the highest contents of non-structural carbohydrates (NSC), consisting mainly of starch (431 and 328 g/Kg DM, respectively), and the lowest contents of cellulose, hemicellulose, and lignin (~382 and ~406 g/Kg DM, respectively). *C. alpina* and *T. vulgare* showed the highest contents of lignin (100 and 108 g/Kg DM, respectively). The content of hemicellulose was calculated to be highest in the CTRL (~260 g/Kg DM), and between 123 and 189 g/kg DM in the alpine herbs.

The content of crude protein was lowest in *Sa. officinalis* (~60 g/Kg DM), highest in *G. odoratum* (~133 g/Kg DM), and between 71 and 98 g/Kg DM for all other alpine herbs. *Sa. officinalis* and *A. vulgaris* had the highest contents of total polyphenols (TP) (32 and 14 g/Kg DM, respectively) and *S. officinalis* and the CTRL had the lowest (0.9 and 1.4 g/Kg DM, respectively). All of the other alpine herbs had approximately 6.2–6.6 g/kg DM of TP.

2.3. Experimental Design and Incubation Procedure

The full procedure of the in vitro cow-rumen experiment was carried on at the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of Padova University, (Legnaro, Padova, Italy).

Three lactating Simmental cows in the experimental farm "Lucio Toniolo" of the University of Padova (Legnaro, Padova, Italy) were chosen as rumen fluid donors. The rumen fluids were collected from the cows by use of an esophageal probe. The collected rumen fluid (approximately 1 L per cow) was maintained at 39.0 °C in a thermos and promptly transported to the laboratory. Upon arrival, it underwent filtration using four layers of cheesecloth to remove coarse particles. All procedures were carried out under anaerobic conditions using a flow of carbon dioxide (CO_2) and were completed in less than 40 min to ensure the preservation of microflora activity. Following the methods of Menke and Steingass [24], artificial buffered inoculum was prepared and then mixed with the rumen fluid at a volume ratio of 2:1 for a total of 150 mL before being kept at 39 °C under the flux of CO_2 for 45 min to maintain anaerobic conditions. Afterward, 1.00 ± 0.01 g of each herb was weighed and added to the fermentation flask. Finally, the bottle headspace

(pre-heated to 39 °C) was filled with nitrogen (N_2), instead of CO_2, to maintain anaerobic conditions and avoid interference with the total-gas and methane quantification [25]. A control bottle (CTRL) supplemented with *Poaceae* grass hay and a bottle (BLANK) with no supplementation were incubated under the same conditions alongside the samples. In vitro ruminal fermentation was performed at 39 °C for 48 h. This setup was repeated in triplicate for three consecutive weeks for a total of 72 in vitro ruminal fermentations (6 herbs × 3 rumen fluids × 3 replicates + 9 BLANKs + 9 CTRLs).

2.4. DNA Extraction, 16S rRNA Gene Amplification, and MiSeq Illumina Sequencing

Total genomic DNA was extracted from 1 mL of each 24 h incubation sample by using the QIAamp PowerFecal DNA kit (Qiagen, Milan, Italy) according to the kit instructions. The yield and purity of the extracted DNA were determined by the Nanodrop8800 fluorospectrometer (Thermo Scientific, Waltham, MA, USA).

Amplicon library preparation, the determination of the quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, San Diego, CA, USA) were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy).

PCR amplification was performed by targeting 16S rRNA gene V3-V4 variable regions [26,27], with the bacterial primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GACTACNVGGGTWTCTAATCC-3′). PCR amplification of each sample was carried out in 25 µL of reaction volume, with 12.5 µL of 2X KAPA Hifi HotStart Ready Mix (Kapa Biosystems Ltd., London, UK), 1 µM of each primer, 2 µL of DNA (10 ng/µL), and 9.5 µL of ddH20. All PCR reactions were carried out using a Verity™ 96-well Thermal Cycler, according to the following protocol: 95 °C for 5 min and 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, with a final elongation step of 72 °C for 5 min. PCR products were checked by gel electrophoresis and cleaned using an Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), following the manufacturer's instructions. After seven PCR cycles (16S metataxonomic Sequencing Library Preparation, Illumina), Illumina adaptors were attached (Illumina Nextera XT Index Primer). Libraries were purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA), and then sequenced on an Illumina® MiSeq (Run Chemistry: 2 × 300 PE) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis software 1.16.18, Illumina, San Diego, CA, USA).

2.5. Illumina Data Analysis and Sequence Identification

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp, last access 28 November 2024) and imported into Quantitative Insights in Microbial Ecology (Qiime2, version 2020.11). Sequences were quality-filtered, trimmed, de-noised, and merged using DADA2 [28]. Chimeric sequences were identified and removed, using the consensus method, in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree, using plugin alignment and phylogeny [29,30]. Taxonomic and compositional analyses were conducted by using the plugin feature-classifier (https://github.com/qiime2/q2-feature-classifier, last access 28 November 2024). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99% operational taxonomic units (OTUs) database (http://greengenes.secondgenome.com/, last access 28 November 2024), which had previously been trimmed to the V4 region of 16S rDNA and bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by Illumina sequencing were deposited in the NCBI Sequence Reads Archive (SRA), BioProject accession number PRJNA1175582

2.6. Nuclear Magnetic Resonance Spectrometry of Rumen Liquid

Nuclear magnetic resonance spectroscopy analysis (NMR) was performed on rumen liquid and rumen liquid mixed with medium solution prior to fermentation (Section 2.5). A quantity of 900 µL of rumen liquid was thoroughly mixed with 100 µL of deuterated water (D_2O, 99.9% isotopic purity containing 0.03% TMSP-d4, Deutero GmbH, Kastellaun, Germany). Samples were centrifuged for 15 min at 12,000 rpm and 600 µL supernatant was filtered using 0.22 µm PVDF filters (Millex-GV, polyvinylidene fluoride membrane, Millipore, Bedford, MA, USA) into 5 mm NMR tubes (509-UP, Norell, Landisville, NJ, USA).

The NMR spectra were recorded with a Bruker Avance Neo 600 spectrometer at a base frequency of 600.13 MHz for protons; the spectrometer was equipped with broadband Z-gradient probe for 5 mm sample tubes and a 24-position refrigerated SampleCase autosampler (Bruker BioSpin GmbH, Rheinstetten, Germany).

Topspin 4.1.4 with Icon NMR 5.2.4 was used to record and process spectra, and a deuterium lock signal was optimized for the 9:1 mixture of H_2O and D_2O (v/v).

The experimental parameters for NMR were as follows: we used a noesygppr1d pulse sequence set with a power level utilized per pulse of 49.51 dB (25 Hz suppression window); the size of the spectrum was 20.83 ppm, the time domain consisted of ~128 K data points, 64 actual scans and 4 dummy scans were performed, the time for relaxation delay was 10 s, the receiver gain for spectra was fixed at 16, and the baseopt mode was used for digitization. Before each measurement, automatic adjustment of the probe and automatic shimming were performed. Each spectrum was automatically processed using the TopSpin software and the apk0.noe phase-correction program.

Analysis was performed using the AssureNMR software v. 2020.09.23 [31] using the external standard method of the ERETIC technique (electronic reference to access in vivo concentrations) based on PULCON (pulse length-based concentration determination principle) [32,33]. As a standard sample, a 2 mmol sucrose solution in water, supplied by the manufacturer, was analyzed alongside the samples. This standard was also used for validation by comparing it against another standard (20 mmol sucrose and hippuric acid, also in water) to evaluate the accuracy of measurement [34].

Compounds were identified using the automation mode in AssureNMR, utilizing the Human Metabolome Database [35] and the BBIOREFCODE database of NMR metabolites (v.2.01, Bruker BioSpin GmbH, Rheinstetten, Germany). Any unidentified peaks were identified manually using the previous literature as reference [36,37].

The contents of the compounds of rumen liquid collected prior to fermentation are shown in Supplementary Table S1. The contents were compared between different cows and between the different weeks, as these factors can impact the rumen environment [38,39]. Using the Kruskal–Wallis U test, we did not detect any significant differences in the contents of the compounds in the rumen between the rumen of different cows or among different weeks.

2.7. Statistics

Alpha-diversity determination was performed with the observed OTUs number and Shannon diversity index, and statistical significance between groups was evaluated by the Kruskal–Wallis H test in QIIME2; beta-diversities were calculated using the unweighted and weighted dissimilarity distance matrix in QIIME2. The beta-diversity distance matrix indicates differences in taxa composition between samples based on either presence–absence or quantitative species abundance data. The output matrix was ordinated using principal coordinate analysis (PCoA) and visualized using EMPeror [40]. Statistical significance of the beta-diversity distances between groups was assessed using PERMANOVA with 999 permutations in QIIME2.

Statistical significance of the concentrations of compounds of rumen fluid and the ratios of Firmicutes/Bacteroidetes between fermentations were tested using the Kruskal–Wallis *H*-test with the associated Dunn test, performed using XLSTAT version 2024.3.0. Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Composition of In Vitro Rumen Fermentation Microbiota

The relative abundances of bacterial phyla of the in vitro rumen fermentations are shown in Table 1 and Supplementary Figure S1A. The dominant phyla of all RF samples were Bacteroidetes and Firmicutes, with Bacteroidetes generally being the most abundant of the two. The herbs and CTRL samples showed Bacteroidetes relative abundance to be in the range of 47–58%. A higher abundance of Bacteroidetes associated with a lower abundance of Firmicutes was observed for RF supplemented with Cic, San, and Tan, which are alpine herbs with high contents of lignin. As Bacteroidetes are generally more efficient in degrading fiber than are Firmicutes [41], this could suggest the presence of a more efficient ruminal fermentation when these alpine herbs are present in the cow diet. In all the RF supplemented with herbs, the relative abundance of Firmicutes was similar (30.9–39.7%), except for RF-Cic, in which Firmicutes relative abundance was the lowest (19.84%).

The ratio of Firmicutes/Bacteroidetes was not significantly different among the RF with a supplemented herb. The highest ratio was recorded in RF-Gal and the lowest in RF-Cic. A higher Firmicutes/Bacteroidetes ratio in the rumen is associated with an increased milk-fat yield in lactating cows [8], suggesting that feeding cows with *G. odoratum* could increase the milk yield, and by the converse, feeding with *C. alpina* could decrease it.

Besides Firmicutes and Bacteroidetes, the only phyla with an abundance > 10.0% were Proteobacteria and Fibrobacteres. The highest relative abundance of Proteobacteria was observed in RF-San samples, in which the Proteobacteria presence has been correlated with the high content of tannins [13]. The highest relative abundance of Fibrobacteres was observed in RF-Cic and RF-Sis samples, and the lowest in RF-Alc and RF-Tan samples. The other phyla with a relative abundance > 1.0% were the Tenericutes, Planctomycetes, Spyrochaetes, SR1, and TM7 phyla. Although they have previously been observed in the rumen, their role has not been fully explored [42–45].

Lastly, the Actinobacteria, Chloroflexi, Cyanobacteria, Elusimicrobia, Fusobacteria, Lentisphaerae, Synergistetes, Verrucomicrobia, and Archaea phyla always showed a relative abundance lower than 1.0%. These phyla are commonly observed in the rumen and some have been associated with benefits to the rumen fermentation and/or cow health [46–51]. Due to the low abundance observed in this study we cannot confidently predict whether their presence is significantly associated with the herb material supplemented in the in vitro fermentations.

The microbial composition of the in vitro RF at the taxa level is represented in Table 1 and Supplementary Figure S1B, which show only the taxa with a relative abundance > 1% in at least one sample. The taxonomic groups with the highest relative abundance across all RF were *Prevotella*, *Bacteroidales*, and *Clostridia*. Compared to the CTRL, RF with alpine herbs showed in all cases higher abundances of *Prevotella* and *Gammaproteobacteria* and lower abundances of bacterial taxa belonging to the *Clostridia* group, such as *Butyrivibrio*, *Ruminococcaceae*, *Anaerovibrio*, *Succiniclasticum*, and *Desulfovibrio* taxa.

Table 1. Relative abundance, expressed in %, of bacterial phyla and taxa of in vitro rumen fermentation, based on Illumina Miseq identification. Composition of taxa is reported as relative abundance % and ratios as the mean ± standard deviation. Grey squares represent the relative abundances ≥ 1.0% and dark grey squares represent the relative abundances ≥ 10.0%.

Phyla	**Rumen**	**Blank T0**	**Blank T24**	**CTRL**	**RF-Alc**	**RF-Cic**	**RF-Gal**	**RF-San**	**RF-Sis**	**RF-Tan**
Archaea	0.48	0.34	0.48	0.60	0.18	0.083	0.099	n.d.	0.38	0.21
Actinobacteria	n.d.	0.69	0.28	0.10	n.d.	0.044	0.036	0.085	n.d.	0.26
Bacteroidetes	73.022	66.13	29.51	49.95	52.61	57.92	46.99	52.12	48.28	54.62
Chloroflexi	n.d.	n.d.	0.84	0.29	0.089	0.18	0.15	0.11	0.12	0.42
Cyanobacteria	0.13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.073	n.d.	n.d.
Elusimicrobia	n.d.	0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fibrobacteres	0.18	n.d.	0.18	3.83	0.49	10.73	2.06	1.48	9.20	0.07
Firmicutes	19.71	23.48	44.66	33.60	37.37	19.84	39.67	30.93	31.24	34.94
Fusobacteria	n.d.	n.d.	n.d.	n.d.	n.d.	0.10	n.d.	n.d.	n.d.	0.039
Lentisphaerae	0.13	n.d.	n.d.	0.044	0.12	n.d.	0.036	0.043	n.d.	n.d.
Planctomycetes	0.97	1.75	1.60	1.93	0.45	0.57	1.02	0.43	0.88	1.20
Proteobacteria	1.88	3.56	21.25	6.23	5.10	6.31	6.20	11.08	6.64	6.30
SR1 [1]	2.27	2.40	0.85	0.82	0.86	2.07	0.79	0.35	2.16	1.56
Spirochaetes	0.22	0.11	0.01	1.69	0.89	0.70	0.74	1.15	0.75	0.08
Synergistetes	n.d.	n.d.	0.13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TM7 [2]	0.50	1.30	0.20	0.84	n.d.	n.d.	0.10	0.02	0.34	0.26
Tenericutes	0.50	0.13	0.023	0.053	1.10	1.34	2.13	1.59	n.d.	0.047
Verrucomicrobia	n.d.	n.d.	n.d.	n.d.	0.75	0.12	n.d.	0.54	n.d.	n.d.
Firmicutes/ Bacteroidetes	0.28 ±0.13 [A]	0.37 ±0.15 [A]	2.05 ±1.81 [B]	0.72 ±0.30 [AB]	0.81 ±0.44 [AB]	0.37 ±0.20 [A]	0.89 ±0.30 [AB]	0.62 ±0.20 [AB]	0.73 ±0.34 [AB]	0.81 ±0.68 [AB]
Taxa	Rumen	Blank T0	Blank T24	CTRL	RF-Alc	RF-Cic	RF-Gal	RF-San	RF-Sis	RF-Tan
Bacteroidetes; family *BS1* [3]	1.75	1.16	1.35	3.33	0.81	1.87	1.41	0.94	1.70	2.41
Bacteroidetes; *Prevotella*	34.943	31.163	5.143	16.060	21.640	29.330	27.246	26.607	19.875	27.179
Bacteroidetes; *Paraprevotellaceae*	1.486	1.352	0.347	1.514	1.476	3.606	3.419	3.486	1.809	0.900
Bacteroidetes; family *RF16* [3]	5.99	0.76	7.64	1.49	5.010	1.70	1.82	4.086	1.97	0.62
Other *Bacteroidales*	28.630	31.608	15.018	27.506	23.674	21.412	15.645	16.895	22.862	23.505
Other *Bacteroidetes*	0.224	0.091	0.007	0.049	n.d.	n.d.	n.d.	0.101	0.067	0.009
Fibrobacteres; *Fibrobacter succinogenes*	0.18	n.d.	0.18	3.83	0.22	10.73	2.061	1.058	9.20	0.069

Table 1. *Cont.*

Phyla	Rumen	Blank T0	Blank T24	CTRL	RF-Alc	RF-Cic	RF-Gal	RF-San	RF-Sis	RF-Tan
Firmicutes: *Lysinibacillus*	n.d.	n.d.	10.103	n.d.	0.019	0.293	0.411	0.088	n.d.	0.013
Firmicutes: *Rummeliibacillus*	n.d.	n.d.	1.893	n.d.	n.d.	0.057	n.d.	n.d.	0.339	2.706
Firmicutes: *Solibacillus*	n.d.	n.d.	4.691	n.d.	n.d.	n.d.	n.d.	0.091	n.d.	n.d.
Firmicutes: *Planococcaceae*	n.d.	n.d.	14.684	n.d.	4.653	0.191	n.d.	n.d.	n.d.	n.d.
Firmicutes: *Streptococcus*	n.d.	n.d.	n.d.	2.581	11.738	0.675	7.325	13.581	9.467	1.143
Firmicutes: *Butyrivibrio*	2.941	3.964	0.844	5.269	1.684	1.043	1.820	2.598	3.028	4.420
Firmicutes: other *Lachnospiraceae*	1.169	1.057	1.875	5.038	4.984	6.164	20.575	6.683	6.221	18.434
Firmicutes: *Ruminococcaceae*	6.597	6.700	2.763	4.615	2.665	3.121	2.137	1.434	4.070	2.063
Firmicutes: *Anaerovibrio*	0.191	0.170	0.571	1.359	0.460	0.699	n.d.	n.d.	0.440	0.224
Firmicutes: *Selenomonas ruminantium*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.111	1.209	0.373	0.145
Firmicutes: *Succiniclasticum*	5.244	6.993	2.490	6.228	4.449	3.493	1.250	2.858	1.540	1.465
Other *Clostridia*	3.340	4.585	4.709	7.791	6.137	3.915	2.469	2.010	4.735	4.333
Other *Firmicutes*	0.230	0.009	0.036	0.148	0.319	0.188	0.236	0.069	0.190	n.d.
Betaproteobacteria; *Comamonadaceae*	n.d.	n.d.	6.632	0.163	n.d.	2.736	0.178	2.869	0.516	0.701
Other *Betaproteobacteria*	n.d.	n.d.	6.263	n.d.	0.107	n.d.	0.036	n.d.	n.d.	0.098
Desulfovibrionaceae; *Desulfovibrio*	n.d.	0.143	1.247	4.318	0.455	1.350	1.074	0.601	2.654	1.476
Other *Deltaproteobacteria*	0.682	1.580	0.498	1.473	3.604	1.118	3.231	4.365	1.949	2.343
Gammaproteobacteria; *Succinivibrionaceae*	n.d.	0.073	n.d.	n.d.	0.284	0.408	1.506	1.401	0.841	0.233
Gammaproteobacteria; *Enterobacteriaceae*	n.d.	n.d.	1.700	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gammaproteobacteria; *Moraxella*	n.d.	n.d.	1.389	n.d.	n.d.	0.087	n.d.	n.d.	n.d.	n.d.
Gammaproteobacteria; *Pseudomonas*	1.006	1.733	3.146	0.078	0.222	0.560	0.738	1.485	0.667	0.299
Other *Gammaproteobacteria*	0.504	0.129	0.023	0.053	1.105	1.340	1.220	1.591	n.d.	0.047

[1] SR1 is a well characterized but uncultured phyla with the candidate name Candidatus Absconditabacteria. [2] TM7 is a well characterized but uncultured phyla with the candidate name Candidatus Saccharibacteria. [3] Uncultivated but well-characterised bacteria. n.d.: Not detected, or a relative abundance < 0.001%. Ratio values with different capital letters in superscript are significantly different.

The *Bacteroidetes* phylum was mainly represented by *Prevotella* and *Paraprevotellaceae*. We observed RF-Cic, RF-Gal, and RF-Tan to have the highest relative abundance of *Prevotella* while RF-Cic, RF-Gal and RF-San showed the highest abundance of *Paraprevotellaceae*.

Species belonging to the *Prevotella* genus are versatile fibrolytic bacteria known to degrade plant hemicellulose, pectin, and proteins [7,8,47,48], and are part of the core microbiome of the rumen environment [18]. Their abundance could correlate with improved RF.

The family *Bacteroidales* RF16 has been associated with digestion of plant fibre [48], and was observed in higher abundance for RF-Alc and RF-San.

The *Bacteroidales* family BS11 has been shown to have hemicellulolytic activity in the rumen [50], and was found with the highest relative abundance in the CTRL and RF-Tan and with the lowest abundance in RF-Alc.

High relative abundances have been assigned no more accurately than as being of the *Bacteroidales* order. Much of the rumen microbiota is still uncharacterized [8], but *Bacteroidales* are known as part of the core ruminal microbiota [18], along with *Paraprevotellaceae* [51]. *Paraprevotellaceae* were observed to be highest in RF-Cic, RF-Gal, and RF-San RF.

The Firmicutes phylum mainly consisted of *Streptococcus*, in addition to *Clostridia* taxa such as *Lachnospiraceae*, *Butyrivibrio*, *Ruminococcaceae*, and *Succiniclastum*.

The highest abundance of *Streptococcus* was observed in RF-Alc and RF-San. The lowest abundance (<1.0%) was observed in RF-Cic. *Streptococcus* is a genus known to ferment starch and its high presence could be associated to subacute ruminal acidosis [52]. Although high relative abundance of *Streptococcus* was observed in some RF, no acidity increase was found to be associated to the RF in the batches, indicating that the increase of *Streptococcus* abundance was not such that would affect the 24 h RF [22].

The Clostridia class is part of the core ruminal microbiota [18], and the highest abundance of total Clostridia bacteria (28–31%) was observed in the CTRL, RF-Gal, and RF-Tan, and the lowest (17–18%) in RF-San and RF-Cic. The higher abundance of Clostridia was mainly attributed to the *Lachnospiraceae* group (*Butyrivibrio* and other taxa) in RF-Gal (22.40%) and RF-Tan (22.85%). *Lachnospiraceae* are part of the core rumen microbiota, and associated with a pasture-based diet [7,18,53] and in the rumen, are involved in cellulose and protein degradation [54]. In the CTRL, the relative abundance of *Lachnospiraceae* was 10.31% and the Clostridia group was mainly represented by taxa present in lower amounts (or not present at all) in the other samples such as *Ruminococcaceae* (4.62%), *Anaerovibrio* (1.36%), and *Succiniclasticum* (6.23%).

Bacteria belonging to the *Butyrivibrio* genus are able to ferment cellulose, hemicellulose, pectin, and proteins [7,47,48] and are the main bio-hydrogenating bacteria in the rumen [7]. The relative abundance of *Butyrivibrio* was highest in the CTRL and RF-Tan.

The CTRL and RF-Sis showed the highest relative abundances of *Ruminococcaceae* known to be both cellulolytic and hemicellulolytic [55,56]. The CTRL and RF-Sis showed the higher values of cellulose and hemicellulose suggesting these substrates could shift the microbiota towards higher abundances of *Ruminococcaceae*.

Some *Lachnospiraceae* genera have been associated with efficient RF; by contrast, *Butyrivibio* has been associated with inefficiency [11,12]. RF-Gal showed a high *Lachnospiraceae* relative abundance, but low *Butyrivibio* relative abundance, suggesting *Galium odoratum* as an alpine herb able to improve RF efficiency when supplemented.

In the rumen, the bacteria belonging to *Anaerovibrio*, *Selenomonas*, and *Succiniclasticum* taxa are propionate producers with respect to the reduction of methane production [7], and are found in higher abundance in the rumen of cows at free pasture compared to animals fed with the conventional mixed feed [53]. The relative abundances of *Anaerovibrio* and *Succiniclasticum* were higher in the CTRL. *Anaerovibrio* are known to have lipolytic activities in the rumen [7], and were observed at a relative abundance > 1.0% only in the

CTRL samples. *Succiniclasticum* is a genus associated with high-starch diets and NCS fermentation [57]. *Selenomonas ruminantium* is an amylolytic bacteria often found in the rumen [50]. Its relative abundance was higher than 1.0% only in RF-San. The alpine herb *Sa. officinalis* had the highest content of polyphenols, which could have selected the population of *Selenomonas* in the RF-San, since *S. ruminnatum* has been found to be tolerant of polyphenols and as having tannin-degrading properties [58].

Planococcaceae and *Lysinibacillus* are non-Clostridia taxa and were mainly observed in Blank T24. They are fibrolytic ruminal bacteria [59,60], and we speculated that their higher abundances in Blank T24 could be caused by the lower abundances of other fibrolytic bacteria such as *Prevotella.* Other functions of these bacteria in the rumen are currently unknown [61,62].

Fibrobacteres relative abundance was highest in RF-Cic and RF-Sis and lowest (<1.0%) in RF-Alc and RF-Tan. *Fibrobacteres* were mainly represented by *Fibrobacter succinogenes*, which is capable of utilizing both cellulose and pectin as substrate in the rumen [62,63].

No taxa belonging to Proteobacteria phylum were present at >10.0%; they mainly consisted of *Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria*.

Comamonadaceae, *Enterobacteriaceae*, *Moraxella*, and *Pseudomonas* were observed mainly in Blank T24, and *Comamonadaceae* were observed with a relative abundance higher than 1.0% only in RF-Cic and RF-San. Although they have previously been observed in the rumen, their roles have yet to be found [64–66].

Desulfovibrio relative abundance was highest in the CTRL and RF-Sis and lowest in RF-Alc and RF-San. *Desulfovibrio* is a major sulphate-reducing bacteria in the rumen and has been shown to increase with sulphur content in diet [67].

All RF samples showed the presence of *Succinivibrionaceae*, except for the CTRL. *Succinivibrionaceae* are associated with a high-starch diet and increased feed efficiency [36] and compete with methanogens for the use of hydrogen [63]; they were higher in RF-Gal and RF-San, confirming the role of *Galium odoratum* in increasing rumen fermentation efficiency when supplemented

The polyphenol contents of *A. vulgaris* and *Sa. officinalis* predominantly consist of condensed tannins [68–70] and their RF showed higher relative abundances of *Streptococcus* and *Bacteroidales* family RF16 and lower abundances of *Bacteroidales* family BS11 and *Desulfovibrio*. A high content of tannins can inhibit the rumen fermentation [71], but can also reduce the methane production of the rumen [72,73] possibly by shifting ruminal metabolism toward propionate production [74].

Archaea were detected in all the samples, except for RF-San. Archaea; the abundance in all RF samples was lower than in CTRL samples. Archaea are the primary producers of methane in the cow rumen [75,76]. The lower abundance of Archaea may therefore be a sign of a lowered methane production following the feeding with alpine herbs.

Feeding strategies can reduce methane production by a modulation of the rumen microbiota. Methane mitigation strategies often focus on inhibiting the growth or activity of Archaea methanogens, which are the main drivers of methane production [54,76,77]. Stimulating competition with methanogens is a strategy gaining traction [75,76], as mitigating methane production through H_2 competition avoids accumulation of the gas in the rumen, which can inhibit fermentation and thereby feed efficiency [10]. A competitive use of hydrogen can occur by propionate production by bacteria such as *Prevotellaceae* and *Succinivibrionaceae* [78–81], by lactate production *Streptococcus* [74], or by sulphate reduction by bacteria such as *S. ruminantium* [82].

In our study, higher abundances of *Streptococcus* were in RF-San, RF-Alch, and RF-Sis; *S. ruminantium* was higher in RF-San; and the propionate producers *Prevotellaceae* and

Succinivibrionaceae were higher in RF-San and RF-Gal, suggesting that except for *Tanacetum vulgare*, all alpine herbs could have some positive effect in methane production reduction.

3.2. Microbial Diversity of In Vitro Rumen Fermentation

To evaluate differences between the bacterial microbiota from alpine herbs and *Poaceae* grass hay (CTRL) within in vitro RF, comparative analyses were performed with the sequences generated in this study. The microbial richness (observed OTUs number) and diversity (Shannon index) were compared between alpine herbs and *Poaceae* grass hay (Table 2). Based on both the number of observed OTUs and the Shannon index, the in vitro rumen fermentations without supplementations (Blank T24) contained the highest level of bacterial richness and diversity. The fermentations with the highest microbial richness were RF-Alc and RF-Cic, and those of the highest diversity were RF-Cic, RF-Sis, and RF-Tan.

Table 2. Richness expressed as observed OTU number (Obs OTUs) and diversity expressed by Caho1, Shannon, and Evenness indices of the bacterial communities identified by 16S amplicon sequencing of the alpine herbs and CTRL in vitro rumen fermentations. Results are shown as mean values and standard deviations (SD) of 9 values.

	Observed OTUs	Shannon	Caho1	Evenness
Rumen	85 ± 22^{AB}	0.586 ± 0.023^{B}	87 ± 26^{AB}	0.931 ± 0.0185^{B}
Blank T0	70 ± 16^{AB}	0.572 ± 0.033^{B}	70 ± 16^{AB}	0.937 ± 0.079^{B}
Blank T24	102 ± 29^{B}	0.594 ± 0.045^{B}	108 ± 36^{B}	0.903 ± 0.0163^{A}
CTRL	79 ± 26^{AB}	0.517 ± 0.191^{AB}	79 ± 26^{AB}	0.932 ± 0.0084^{B}
RF-Alc	80 ± 13^{AB}	0.501 ± 0.182^{A}	81 ± 14^{AB}	0.923 ± 0.0127^{AB}
RF-Tan	65 ± 9^{A}	0.547 ± 0.013^{AB}	65 ± 9^{A}	0.902 ± 0.0165^{A}
RF-Gal	66 ± 20^{AB}	0.477 ± 0.177^{A}	67 ± 22^{AB}	0.903 ± 0.0135^{A}
RF-San	64 ± 12^{A}	0.479 ± 0.175^{A}	64 ± 12^{A}	0.923 ± 0.0092^{AB}
RF-Sis	63 ± 12^{A}	0.549 ± 0.029^{AB}	63 ± 12^{A}	0.937 ± 0.0085^{B}
RF-Cic	89 ± 23^{AB}	0.532 ± 0.192^{AB}	90 ± 25^{AB}	0.913 ± 0.0211^{AB}

For each variable (Obs OTUs, Shannon, caho1, and evenness), values with different superscript letters are significantly different ($p < 0.05$, one-way Anova with post hoc Tukey HSD).

A lower microbial diversity in the rumen is associated with a more efficient fermentation [11], but a higher diversity increase the redundancy and thereby the robustness of the rumen microbiota [7]. Therefore, the lower diversity of RF-Gal, RF-San, and RF-Alc may suggest a higher efficiency but a lower robustness of the microbiota.

Two distance matrices were created based on weighted and unweighted UniFrac indices; these were used to calculate distances between pairs of samples, representing how closely related those samples were. The PCoA based on weighted (Figure 1a) and unweighted (Figure 1b) UniFrac distance matrices showed similar results. The first two axes explained 51.20% and 19.55% of the variance of data based on the weighted and unweighted distance matrix, respectively. Samples were colored according to the supplementation of the rumen fermentations. Samples closer to one another are more similar than those that are further away from each other.

Considering the weighted UniFrac distance matrix, the Blank rumen samples at T0 (black and blue) were separate from all other fermentations. Among RF samples with alpine herbs, only RF-Gal and RF-Tan were clearly separate along the second component. Other samples are spread across the figure.

The unweighted UniFrac distance accounts for the presence/absence of OTUs, whereas weighted UniFrac accounts for the abundance as well. The greatest separation was observed for the weighted UniFrac distance matrix and accounts for a higher variance fraction, suggesting that both abundance and the presence/absence of OTUs are important for characterization of microbiota in the different rumen fermentations with alpine herbs.

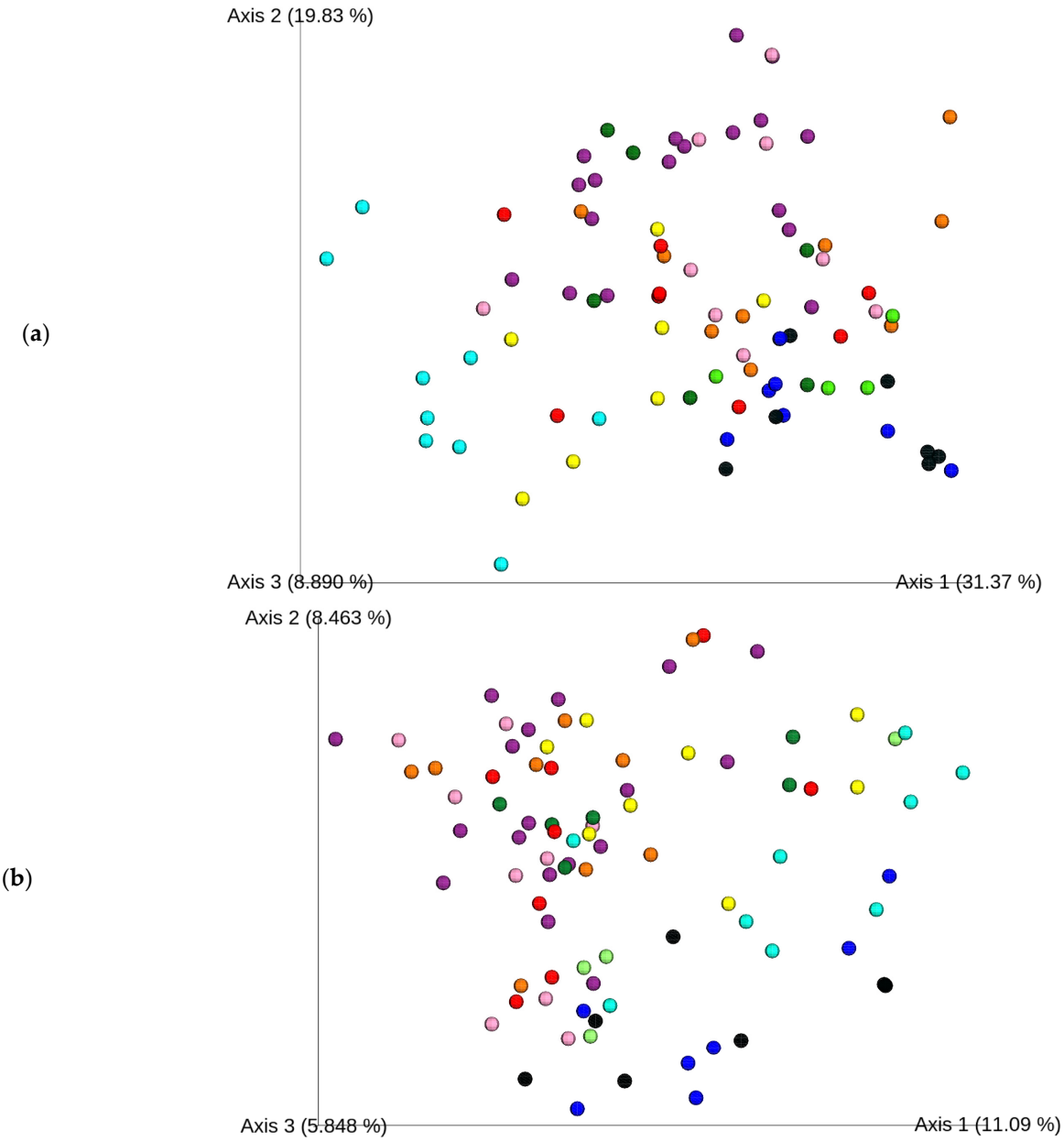

Figure 1. Beta-diversity microbiota changes based on supplementation with herbs during rumen fermentations. A principal coordinate analysis (PCoA) ordination using weighted (**a**) and unweighted (**b**) distances was performed to visualize microbial community OTU differences across the different fermented herbs. Rumen: black; Blank T0: blue; Blank T24: light blue; CTRL: yellow; RF-Alc: red; RF-Cic: orange; RF-Gal: purple; RF-San: pink; RF-Sis: dark green; and RF-Tan: light green. For the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

The PERMANOVA analysis is shown in (Table 3), showing the amount (Pseudo-F) and significance (p) of microbial composition differences between fermentation conditions. We observed higher average and absolute pseudo-F values with the PERMANOVA using weighted UniFrac measurements, compared to unweighted.

The highest and significant differences (highest Pseudo-F values) were recorded between Blank T0 or Rumen when compared to Blank T24.

The RFs of all alpine herbs were significantly different when compared to the CTRL, using both weighted and unweighted UniFrac, except for RF-Sis when using weighted UniFrac.

Table 3. PERMANOVA analysis (999 permutations) results for bacterial communities based on weighted and unweighted UniFrac distances.

Pairwise Comparisons for Herb Fermentation	Weighted UniFrac		Unweighted UniFrac	
	Pseudo-F	*p*-Value	Pseudo-F	*p*-Value
Rumen vs. Blank T0	0.656	0.652	1.109	0.314
Rumen vs. Blank T24	19.340	0.001 **	3.143	0.002 **
Rumen vs. RF-Alc	5.245	0.005 **	2.908	0.002 **
Rumen vs. RF-Cic	5.164	0.003 **	3.038	0.001 **
Rumen vs. RF-Gal	16.420	0.001 **	4.519	0.001 **
Rumen vs. RF-San	6.638	0.001 **	2.876	0.001 **
Rumen vs. RF-Sis	6.270	0.002 **	2.943	0.001 **
Rumen vs. RF-Tan	1.863	0.106	2.208	0.002 **
Rumen vs. CTRL	6.915	0.003 **	2.806	0.001 **
Blank T0 vs. Blank T24	16.405	0.001 **	2.732	0.002 **
Blank T0 vs. RF-Alc	4.290	0.006 **	2.966	0.002 **
Blank T0 vs. RF-Cic	4.967	0.001 **	3.484	0.003 **
Blank T0 vs. RF-Gal	13.385	0.001 **	4.053	0.001 **
Blank T0 vs. RF-San	5.347	0.001 **	2.656	0.001 **
Blank T0 vs. RF-Sis	4.835	0.003 ***	2.814	0.001 ***
Blank T0 vs. RF-Tan	2.124	0.056	1.932	0.008 *
Blank T0 vs. CTRL	5.178	0.001 **	2.820	0.001 **
Blank T24 vs. RF-Alc	9.392	0.001 **	3.114	0.001 **
Blank T24 vs. RF-Cic	16.823	0.002 **	3.450	0.001 **
Blank T24 vs. RF-Gal	22.458	0.001 **	4.755	0.001 **
Blank T24 vs. RF-San	13.150	0.001 **	3.481	0.001 **
Blank T24 vs. RF-Sis	9.056	0.001 **	2.579	0.001 **
Blank T24 vs. RF-Tan	11.966	0.001 **	1.689	0.022 *
Blank T24 vs. CTRL	8.853	0.001 **	2.377	0.002 **
RF-Alc vs. RF-Cic	4.299	0.010 *	1.740	0.004 **
RF-Alc vs. RF-Gal	4.965	0.002 **	1.878	0.008 **
RF-Alc vs. RF-San	1.619	0.112	1.490	0.043 *
RF-Alc vs. RF-Sis	1.971	0.088	2.172	0.002 **
RF-Alc vs. RF-Tan	3.298	0.034 *	1.736	0.028 *
RF-Alc vs. CTRL	2.732	0.037 *	2.307	0.002 **
RF-Cic vs. RF-Gal	8.310	0.001 **	2.004	0.002 **
RF-Cic vs. RF-San	3.884	0.005 **	1.650	0.007 **
RF-Cic vs. RF-Sis	1.574	0.193	1.356	0.090
RF-Cic vs. RF-Tan	2.852	0.057	2.049	0.007 *
RF-Cic vs. CTRL	5.205	0.004 **	2.080	0.002 **
RF-Gal vs. RF-San	4.447	0.001 **	1.914	0.001 **
RF-Gal vs. RF-Sis	4.056	0.003 **	2.133	0.002 **
RF-Gal vs. RF-Tan	7.179	0.001 **	1.767	0.005 **
RF-Gal vs. CTRL	8.440	0.001 **	2.510	0.001 **
RF-San vs. RF-Sis	2.548	0.020 *	2.548	0.016 *
RF-San vs. RF-Tan	3.451	0.007 **	3.451	0.004 **
RF-San vs. CTRL	5.590	0.001 **	2.507	0.001 **
RF-Sis vs. RF-Tan	3.698	0.022 *	1.906	0.001 **
RF-Sis vs. CTRL	1.661	0.155	1.639	0.010 *
RF-Tan vs. CTRL	4.358	0.013 *	1.711	0.029 *

Significance levels: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

RF-Gal showed the highest Pseudo-F values, suggesting the highest differences in terms of rumen microbiota; contrastingly, RF-Sis and RF-Alc were similar and demonstrated the lowest level of difference when compared with the CTRL (lowest pseudo-F values).

Samples and the CTRL RF, compared to Blanks, showed high and significant difference; from largest to the smallest difference, the order was as follows: RF-Gal, RF-Cic, RF-San, RF-Tan, RF-Alc, RF-Sis, and CTRL.

For certain pairs (Rumen and RF-Tan, RF-Alc and RF-San, CTRL and RF-Sis, RF-Sis and RF-Alc, RF-Cic and RF-Tan, and Blank T0 and RF-Tan) no significant difference was

observed by means of weighted UniFrac distance matrix, but the comparison became significant when using an unweighted UniFrac distance matrix. This indicates that the presence/absence of OTUs was more impactful than their abundance for the difference between the pairs.

Considering the alpine herb composition, the most distinctive character of *G. odoratum* when compared to CTRL herbs is the very high value determined for protein.

4. Conclusions

In this study the effect on microbial microbiota of the addition of alpine herbs to in vitro rumen fermentation was examined. Herbs with different compositions of cellulose, hemicellulose, lignin, protein, starch and polyphenols were tested.

The main differences observed for the microbiota on the phyla level was a low abundance of *Firmicutes* in RF-Cic, a higher abundance of *Fibrobacteres* in RF-Cic and RF-Sis, and a higher abundance of *Proteobacteria* in RF-San. The ratio of Firmicutes/Bacteroidetes was not significantly different between alpine herb RF and the CTRL, although RF-Cic did have a noticeably lower ratio.

The different herbs promoted the abundance of various fibrinolytic bacteria with a variety of substrates. Contents of different carbohydrates, protein, lignin, and polyphenols were associated with the changes in microbiota.

The addition of alpine herbs altered the diversity and richness of the microbiota in fermentations; herbs that had a high content of structural fiber resulted in higher diversity, indicating a more robust microbiota, while herbs rich in starch led to lower diversity, suggesting higher efficiency. Beta diversity analyses showed that fermentations with alpine herbs containing similar levels of protein, lignin, and/or polyphenols exhibited less variation in microbiota composition. Differences in alpha diversity were influenced by the content of structural and non-structural carbohydrates in the plant material, while beta diversity between fermentations was primarily determined by the levels of protein, lignin, and polyphenols.

Previous publications have shown tannins can reduce methane production in the rumen. In present study we have data confirming that the tannin-rich *A. vulgaris* and *Sa. officinalis* induced higher abundances of *Streptococcus*, *S. ruminantium*, *Succinivibrionaceae*, and *Prevotellaceae*, which can act as competitors for hydrogen with methanogen archaea, reducing methane production. *Galium odoratum* seems to drive towards higher abundance of *Succinivibrio* and lower values for *Butyrivibrio* microbiota, increasing rumen fermentation efficiency when supplemented.

Supplement of alpine herbs to conventional indoor winter diets could improve dairy production by increasing efficiency or reducing methane production, but the effects observed in this study need to be confirmed in vivo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11020083/s1. Figure S1: Relative abundance of microbiota of an in vitro rumen fermentation, based on Illumina Miseq identification, and shown graphically as stack plot. Table S1: Contents of the rumen liquid before fermentation, as determined by NMR.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data generated by Illumina sequencing were deposited in the NCBI Sequence Reads Archive (SRA), BioProject accession number PRJNA1175582. Other data presented in this study are available on request from the corresponding author.

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References

1. Palumbo, F.; Squartini, A.; Barcaccia, G.; Macolino, S.; Pornaro, C.; Pindo, M.; Sturaro, E.; Ramanzin, M. A multi-kingdom metabarcoding study on cattle grazing Alpine pastures discloses intra-seasonal shifts in plant selection and faecal microbiota. *Sci. Rep.* **2021**, *11*, 889. [CrossRef]
2. Formaggioni, P.; Malacarne, M.; Franceschi, P.; Zucchelli, V.; Faccia, M.; Battelli, G.; Brasca, M.; Summer, A. Characterisation of Formaggella della Valle di Scalve cheese produced from cows reared in valley floor stall or in mountain pasture: Fatty acids profile and sensory properties. *Foods* **2020**, *9*, 383. [CrossRef]
3. Secchi, G.; Amalfitano, N.; Carafa, I.; Franciosi, E.; Gallo, L.; Schiavon, S.; Sturaro, E.; Tagliapietra, F.; Bittante, G. Milk metagenomics and cheese-making properties as affected by indoor farming and summer highland grazing. *J. Dairy Sci.* **2023**, *106*, 96–116. [CrossRef] [PubMed]
4. Carafa, I.; Navarro, I.C.; Bittante, G.; Tagliapietra, F.; Gallo, L.; Tuohy, K.; Franciosi, E. Shift in the cow milk microbiota during alpine pasture as analyzed by culture dependent and highthroughput sequencing techniques. *Food Microbiol.* **2020**, *91*, 103504. [CrossRef]
5. Endrizzi, I.; Cliceri, D.; Menghi, L.; Aprea, E.; Gasperi, F. Does the 'mountain pasture product' claim affect local cheese acceptability? *Foods* **2021**, *10*, 682. [CrossRef]
6. O'Callaghan, T.; Vázquez-Fresno, R.; Serra-Cayuela, A.; Dong, E.; Mandal, R.; Hennessy, D.; McAuliffe, S.; Dillon, P.; Wishart, D.S.; Stanton, C.; et al. Pasture Feeding Changes the Bovine Rumen and Milk Metabolome. *Metabolites* **2018**, *8*, 27. [CrossRef] [PubMed]
7. Palmonari, A.; Federiconi, A.; Formigoni, A. Animal board invited review: The effect of diet on rumen microbial composition in dairy cows. *Animal* **2024**, *18*, 101319. [CrossRef] [PubMed]
8. Cammack, K.M.; Austin, K.J.; Lamberson, W.R.; Conant, G.C.; Cunningham, H.C. Tiny but mighty: The role of the rumen microbes in livestock production. *J. Anim. Sci.* **2018**, *96*, 752–770. [CrossRef]
9. Li, F.; Li, C.; Chen, Y.; Liu, J.; Zhang, C.; Irving, B.; Fitzsimmons, C.; Plastow, G.; Guan, L.L. Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* **2019**, *7*, 92. [CrossRef]
10. Ungerfeld, E.M. Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Front. Microbiol.* **2020**, *11*, 589. [CrossRef]
11. Shabat, S.K.B.; Sasson, G.; Doron-Faigenboim, A.; Durman, T.; Yaacoby, S.; Berg Miller, M.E.; A White, B.; Shterzer, N.; Mizrahi, I. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* **2016**, *10*, 2958–2972. [CrossRef] [PubMed]
12. Fonseca, P.A.S.; Lam, S.; Chen, Y.; Waters, S.M.; Guan, L.L.; Cánovas, A. Multi-breed host rumen epithelium transcriptome and microbiome associations and their relationship with beef cattle feed efficiency. *Sci. Rep.* **2023**, *13*, 16209. [CrossRef]
13. Rira, M.; Morgavi, D.P.; Popova, M.; Maxin, G.; Doreau, M. Microbial colonisation of tannin-rich tropical plants: Interplay between degradability, methane production and tannin disappearance in the rumen. *Animal* **2022**, *16*, 100589. [CrossRef]
14. Beckett, L. Rumen volatile fatty acid molar proportions, rumen epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies. *J. Dairy Sci.* **2021**, *104*, 8857–8869. [CrossRef]

15. Wang, L.; Zhang, G.; Li, Y.; Zhang, Y. Effects of High Forage/Concentrate Diet on Volatile Fatty Acid Production and the Microorganisms Involved in VFA Production in Cow Rumen. *Animals* **2020**, *10*, 223. [CrossRef]
16. Buitenhuis, B.; Lassen, J.; Noel, S.J.; Plichta, D.R.; Sørensen, P.; Difford, G.F.; Poulsen, N.A. Impact of the rumen microbiome on milk fatty acid composition of Holstein cattle. *Genet. Sel. Evol.* **2019**, *51*, 23. [CrossRef] [PubMed]
17. Garcıa-Gonzalez, R.; Lopez, S.; Fernandez, M.; Bodas, R.; Gonzalez, J.S. Screening the activity of plants and species for decreasing ruminal methane production in vitro. *Anim. Feed. Sci. Technol.* **2008**, *147*, 36–52. [CrossRef]
18. Jayanegara, A.; Marquardt, S.; Kreuzer, M.; Leiber, F. Nutrient and energy content, in vitro ruminal fermentation characteristics and methanogenic potential of alpine forage plant species during early summer: Ruminal fermentation traits of alpine forage plants. *J. Sci. Food Agric.* **2011**, *91*, 1863–1870. [CrossRef]
19. Kapp-Bitter, A.N.; Dickhoefer, U.; Kreuzer, M.; Leiber, F. Mature herbs as supplements to ruminant diets: Effects on in vitro ruminal fermentation and ammonia production. *Anim. Prod. Sci.* **2021**, *61*, 470. [CrossRef]
20. Antonius, A.; Pazla, R.; Putri, E.M.; Alma'i, M.I.; Laconi, E.B.; Diapari, D.; Jayanegara, A.; Ardani, L.R.; Marlina, L.; Purba, R.D.; et al. Effects of herbal plant supplementation on rumen fermentation profiles and protozoan population in vitro. *Vet. World* **2024**, *17*, 1139–1148. [CrossRef]
21. Lima, P.R.; Apdini, T.; Freire, A.S.; Santana, A.S.; Moura, L.M.L.; Nascimento, J.C.S.; Rodrigues, R.T.S.; Dijkstra, J.; Garcez Neto, A.F.; Queiroz, M.A.A.; et al. Dietary supplementation with tannin and soybean oil on intake, digestibility, feeding behavior, ruminal protozoa and methane emission in sheep. *Anim. Feed. Sci. Technol.* **2019**, *249*, 10–17. [CrossRef]
22. Wencelová, M.; Váradyová, Z.; Mihaliková, K.; Čobanová, K.; Plachá, I.; Pristaš, P.; Jalč, D.; Kišidayová, S. Rumen fermentation pattern, lipid metabolism and the microbial community of sheep fed a high-concentrate diet supplemented with a mix of medicinal plants. *Small Rumin. Res.* **2015**, *125*, 64–72. [CrossRef]
23. Massaro, S.; Amalfitano, N.; Andersen, J.; Dallavalle, G.; Nikolić, N.; Bailoni, L.; Currò, S.; Vrhovsek, U.; Franciosi, E.; Tagliapietra, F. Alpine Pasture Herbs Redirected Hydrogen Towards Alternative Sinks, Inhibiting Methane Production: In Vitro Study. *Ital. J. Anim. Sci. Expect.* 2025, *in press*.
24. Menke, K.H.; Steingass, H. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen liquid. *Anim. Feed Sci. Technol.* **1988**, *28*, 7–55.
25. Park, K.Y.; Lee, H.G. Can flushing gas distort the rumen in vitro experiment results? *Anim. Feed. Sci. Technol.* **2022**, *285*, 115203. [CrossRef]
26. Baker, G.C.; Smith, J.J.; Cowan, D.A. Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods* **2003**, *55*, 541–555. [CrossRef] [PubMed]
27. Claesson, M.J.; Wang, Q.; O'Sullivan, O.; Greene-Diniz, R.; Cole, J.R.; Ross, R.P.; O'Toole, P.W. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Res.* **2010**, *38*, e200. [CrossRef]
28. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
29. Price, M.N.; Dehal, P.S.; Arkin, A.P. FastTree: Computing Large Minimum Evolution Trees with Profiles instead of a Distance Matrix. *Mol. Biol. Evol.* **2009**, *26*, 1641–1650. [CrossRef]
30. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [CrossRef]
31. Colson, K.; Hicks, J.M.; Fischer, C. Method and Apparatus for Automated Raw Material Screening. US 8248072, 29 September 2020.
32. Akoka, S.; Barantin, L.; Trierweiler, M. Concentration measurement by proton NMR using the ERETIC method. *Anal. Chem.* **1999**, *71*, 2554–2557. [CrossRef]
33. Watanabe, R.; Sugai, C.; Yamazaki, T.; Matsushima, R.; Uchida, H.; Matsumiya, M.; Takatsu, A.; Suzuki, T. Quantitative nuclear magnetic resonance spectroscopy based on PULCON methodology: Application to quantification of invaluable marine toxin, okadaic acid. *Toxins* **2016**, *8*, 294. [CrossRef] [PubMed]
34. Cullen, C.H.; Ray, G.J.; Szabo, C.M. A comparison of quantitative nuclear magnetic resonance methods: Internal, external, and electronic referencing. *Magn. Reson. Chem.* **2013**, *51*, 705–713. [CrossRef] [PubMed]
35. Wishart, D.S.; Guo, A.; Oler, E.; Wang, F.; Anjum, A.; Peters, H.; Dizon, R.; Sayeeda, Z.; Tian, S.; Lee, B.L.; et al. HMDB 5.0: The Human Metabolome Database for 2022. *Nucleic Acids Res.* **2022**, *50*, D622–D631. [CrossRef] [PubMed]
36. Bica, R.; Palarea-Albaladejo, J.; Kew, W.; Uhrin, D.; Pacheco, D.; Macrae, A.; Dewhurst, R.J. Nuclear Magnetic Resonance to Detect Rumen Metabolites Associated with Enteric Methane Emissions from Beef Cattle. *Sci. Rep.* **2020**, *10*, 5578. [CrossRef] [PubMed]
37. Eom, J.S.; Kim, E.T.; Kim, H.S.; Choi, Y.Y.; Lee, S.J.; Lee, S.S.; Kim, S.H.; Lee, S.S. Metabolomics comparison of rumen fluid and milk in dairy cattle using proton nuclear magnetic resonance spectroscopy. *Anim. Biosci.* **2021**, *34*, 213–222. [CrossRef]
38. Indugu, N.; Vecchiarelli, B.; Baker, L.D.; Ferguson, J.D.; Vanamala, J.K.P.; Pitta, D.W. Comparison of rumen bacterial communities in dairy herds of different production. *BMC Microbiol.* **2017**, *17*, 190. [CrossRef]

39. O'Hara, E.; Kenny, D.A.; McGovern, E.; Byrne, C.J.; McCabe, M.S.; Guan, L.L.; Waters, S.M. Investigating temporal microbial dynamics in the rumen of beef calves raised on two farms during early life. *FEMS Microbiol. Ecol.* **2020**, *96*, fiz203. [CrossRef]
40. Vázquez-Baeza, Y.; Pirrung, M.; Gonzalez, A.; Knight, R. EMPeror: A tool for visualizing high-throughput microbial community data. *Gigascience* **2013**, *2*, 2047-217X-2-16. [CrossRef]
41. Kaoutari, A.E.; Armougom, F.; Gordon, J.I.; Raoult, D.; Henrissat, B. The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat. Rev. Microbiol.* **2013**, *11*, 497–504. [CrossRef] [PubMed]
42. Kim, M. Invited Review—Assessment of the gastrointestinal microbiota using 16S ribosomal RNA gene amplicon sequencing in ruminant nutrition. *Anim. Biosci.* **2023**, *36*, 364–373. [CrossRef]
43. Liu, H.; Jiang, H.; Hao, L.; Cao, X.; Degen, A.; Zhou, J.; Zhang, C. Rumen Bacterial Community of Grazing Lactating Yaks (*Poephagus grunniens*) Supplemented with Concentrate Feed and/or Rumen-Protected Lysine and Methionine. *Animals* **2021**, *11*, 2425. [CrossRef] [PubMed]
44. Wei, Z.; Xie, X.; Xue, M.; Valencak, T.G.; Liu, J.; Sun, H. The Effects of Non-Fiber Carbohydrate Content and Forage Type on Rumen Microbiome of Dairy Cows. *Animals* **2021**, *11*, 3519. [CrossRef]
45. Jewell, K.A.; McCormick, C.A.; Odt, C.L.; Weimer, P.J.; Suen, G. Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. *Appl. Environ. Microbiol.* **2015**, *81*, 4697–4710. [CrossRef]
46. Du, H.; Erdene, K.; Chen, S.; Qi, S.; Bao, Z.; Zhao, Y.; Wang, C.; Zhao, G.; Ao, C. Correlation of the rumen fluid microbiome and the average daily gain with a dietary supplementation of *Allium mongolicum* Regel extracts in sheep1. *J. Anim. Sci.* **2019**, *97*, 2865–2877. [CrossRef]
47. Lilian, M.; Rawlynce, B.; Charles, G.; Felix, K. Potential role of rumen bacteria in modulating milk production and composition of admixed dairy cows. *Lett. Appl. Microbiol.* **2023**, *76*, ovad007. [CrossRef] [PubMed]
48. Lv, F.; Wang, X.; Pang, X.; Liu, G. Effects of supplementary feeding on the rumen morphology and bacterial diversity in lambs. *PeerJ* **2020**, *8*, e9353. [CrossRef]
49. McGovern, E.; Kenny, D.A.; McCabe, M.S.; Fitzsimons, C.; McGee, M.; Kelly, A.K.; Waters, S.M. 16S rRNA Sequencing Reveals Relationship Between Potent Cellulolytic Genera and Feed Efficiency in the Rumen of Bulls. *Front. Microbiol.* **2018**, *9*, 1842. [CrossRef] [PubMed]
50. Solden, L.M.; Hoyt, D.W.; Collins, W.B.; Plank, J.E.; Daly, R.A.; Hildebrand, E.; Beavers, T.J.; Wolfe, R.; Nicora, C.D.; Purvine, S.; et al. New roles in hemicellulosic sugar fermentation for the uncultivated Bacteroidetes family BS11. *ISME J.* **2017**, *11*, 691–703. [CrossRef] [PubMed]
51. Golder, H.M.; Thomson, J.; Rehberger, J.; Smith, A.H.; Block, E.; Lean, I.J. Associations among the genome, rumen metabolome, ruminal bacteria, and milk production in early-lactation Holsteins. *J. Dairy Sci.* **2023**, *106*, 3176–3191. [CrossRef]
52. Asanuma, N.; Hino, T. Regulation of fermentation in a ruminal bacterium, *Streptococcus bovis*, with special reference to rumen acidosis. *Anim. Sci. J.* **2002**, *73*, 313–325. [CrossRef]
53. Zhang, X.; Wang, W.; Wang, Y.; Cao, Z.; Yang, H.; Li, S. Metagenomic and metabolomic analyses reveal differences in rumen microbiota between grass- and grain-fed Sanhe heifers. *Front. Microbiol.* **2024**, *15*, 1336278. [CrossRef] [PubMed]
54. Zaplana, T.; Miele, S.; Tolonen, A.C. *Lachnospiraceae* are emerging industrial biocatalysts and biotherapeutics. *Front. Bioeng. Biotechnol.* **2024**, *11*, 1324396. [CrossRef] [PubMed]
55. Wang, K.; Jiang, M.; Chen, Y.; Huang, Y.; Cheng, Z.; Datsomor, O.; Jama, S.M.; Zhu, L.; Li, Y.; Zhao, G.; et al. Changes in the rumen development, rumen fermentation, and rumen microbiota community in weaned calves during steviol glycosides treatment. *Front. Microbiol.* **2024**, *15*, 1395665. [CrossRef] [PubMed]
56. n, P.; Liu, H.; Zhao, J.; Gu, X.; Wei, X.; Zhang, X.; Ma, N.; Johnston, L.J.; Bai, Y.; Zhang, W.; et al. Amino acids metabolism by rumen microorganisms: Nutrition and ecology strategies to reduce nitrogen emissions from the inside to the outside. *Sci. Total Environ.* **2021**, *800*, 149596. [CrossRef]
57. Hua, D.; Hendriks, W.H.; Xiong, B.; Pellikaan, W.F. Starch and Cellulose Degradation in the Rumen and Applications of Metagenomics on Ruminal Microorganisms. *Animals* **2022**, *12*, 3020. [CrossRef]
58. Takizawa, S.; Asano, R.; Abe, K.; Fukuda, Y.; Baba, Y.; Sakurai, R.; Tada, C.; Nakai, Y. Relationship Between Rumen Microbial Composition and Fibrolytic Isozyme Activity During the Biodegradation of Rice Straw Powder Using Rumen Fluid. *Microbes Environ.* **2023**, *38*, ME23041. [CrossRef] [PubMed]
59. Li, Z.; Wright, A.-D.G.; Liu, H.; Bao, K.; Zhang, T.; Wang, K.; Cui, X.; Yang, F.; Zhang, Z.; Li, G. Bacterial Community Composition and Fermentation Patterns in the Rumen of Sika Deer (*Cervus nippon*) Fed Three Different Diets. *Microb. Ecol.* **2015**, *69*, 307–318. [CrossRef]
60. Téllez Martínez, M.G.; Luis, J.; Bolaños, N. *Lysinibacillus fusiformis*: A novel fibrolytic native strain from the rumen microbiome that increases in vitro digestibility of central agricultural residues. *J. Appl. Anim. Res.* **2023**, *51*, 658–668. [CrossRef]

61. De Menezes, A.B.; Lewis, E.; O'Donovan, M.; O'Neill, B.F.; Clipson, N.; Doyle, E.M. Microbiome analysis of dairy cows fed pasture or total mixed ration diets: Microbiome analysis of rumen contents from dairy cows. *FEMS Microbiol. Ecol.* **2011**, *78*, 256–265. [CrossRef]
62. Grilli, D.J.; Cerón, M.E.; Paez, S.; Egea, V.; Schnittger, L.; Cravero, S.; Escudero, M.S.; Allegretti, L.; Arenas, G.N. Isolation of *Pseudobutyrivibrio ruminis* and *Pseudobutyrivibrio xylanivorans* from rumen of Creole goats fed native forage diet. *Folia Microbiol.* **2013**, *58*, 367–373. [CrossRef]
63. Russell, J.B.; Muck, R.E.; Weimer, P.J. Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen: Bacterial QS and rhizosphere N cycling. *FEMS Microbiol. Ecol.* **2009**, *67*, 183–197. [CrossRef] [PubMed]
64. Weimer, P.J. Degradation of Cellulose and Hemicellulose by Ruminal Microorganisms. *Microorganisms* **2022**, *10*, 2345. [CrossRef] [PubMed]
65. Wei, X.; Ouyang, K.; Long, T.; Liu, Z.; Li, Y.; Qiu, Q. Dynamic Variations in Rumen Fermentation Characteristics and Bacterial Community Composition during In Vitro Fermentation. *Fermentation* **2022**, *8*, 276. [CrossRef]
66. Goel, G.; Puniya, A.K.; Aguilar, C.N.; Singh, K. Interaction of gut microflora with tannins in feeds. *Naturwissenschaften* **2005**, *92*, 497–503. [CrossRef] [PubMed]
67. Wu, H.; Li, Y.; Meng, Q.; Zhou, Z. Effect of High Sulfur Diet on Rumen Fermentation, Microflora, and Epithelial Barrier Function in Steers. *Animals* **2021**, *11*, 2545. [CrossRef]
68. Boroja, T.; Mihailović, V.; Katanić, J.; Stanković, M.S.; Bauer, R. The biological activities of roots and aerial parts of *Alchemilla vulgaris* L. *S. Afr. J. Bot.* **2018**, *116*, 175–184. [CrossRef]
69. Cieslak, A.; Zmora, P.; Matkowski, A.; Nawrot-Hadzik, I.; Pers-Kamczyc, E.; El-Sherbiny, M.; Bryszak, M.; Szumacher-Strabel, M. Tannins from *Sanguisorba officinalis* Affect In Vitro Rumen Methane Production and Fermentation. *J. Anim. Plant Sci.* **2016**, *26*, 54–62.
70. Tu, J.; Li, Q.; Zhou, B. The Tannins from *Sanguisorba officinalis* L. (Rosaceae): A Systematic Study on the Metabolites of Rats Based on HPLC–LTQ–Orbitrap MS2 Analysis. *Molecules* **2021**, *26*, 4053. [CrossRef] [PubMed]
71. Battelli, M.; Colombini, S.; Parma, P.; Galassi, G.; Crovetto, G.M.; Spanghero, M.; Pravettoni, D.; Zanzani, S.A.; Manfredi, M.T.; Rapetti, L. In vitro effects of different levels of quebracho and chestnut tannins on rumen methane production, fermentation parameters, and microbiota. *Front. Vet. Sci.* **2023**, *10*, 1178288. [CrossRef]
72. Khatoon, M.; Patel, S.H.; Pandit, R.J.; Jakhesara, S.J.; Rank, D.N.; Joshi, C.G.; Kunjadiya, A.P. Rumen and fecal microbial profiles in cattle fed high lignin diets using metagenome analysis. *Anaerobe* **2022**, *73*, 102508. [CrossRef]
73. Carrasco, J.M.D.; Cabral, C.; Redondo, L.M.; Viso, N.D.P.; Colombatto, D.; Farber, M.D.; Miyakawa, M.E.F. Impact of Chestnut and Quebracho Tannins on Rumen Microbiota of Bovines. *BioMed Res. Int.* **2017**, *2017*, 9610810. [CrossRef] [PubMed]
74. Lan, W.; Yang, C. Ruminal methane production: Associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Sci. Total Environ.* **2019**, *654*, 1270–1283. [CrossRef]
75. Tan, R.S.G.; Zhou, M.; Li, F.; Guan, L.L. Identifying active rumen epithelial associated bacteria and archaea in beef cattle divergent in feed efficiency using total RNA-seq. *Curr. Res. Microb. Sci.* **2021**, *2*, 100064. [CrossRef]
76. Hook, S.E.; Wright, A.-D.G.; McBride, B.W. Methanogens: Methane Producers of the Rumen and Mitigation Strategies. *Archaea* **2010**, *2010*, 945785. [CrossRef]
77. Mao, S.; Zhang, R.; Wang, D.; Zhu, W. The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. *BMC Vet. Res.* **2012**, *8*, 237. [CrossRef]
78. Liu, K.; Xu, Q.; Wang, L.; Wang, J.; Guo, W.; Zhou, M. The impact of diet on the composition and relative abundance of rumen microbes in goat. *Asian-Australas J. Anim. Sci.* **2016**, *30*, 531–537. [CrossRef] [PubMed]
79. Cui, X.; Wang, Z.; Guo, P.; Li, F.; Chang, S.; Yan, T.; Zheng, H.; Hou, F. Shift of Feeding Strategies from Grazing to Different Forage Feeds Reshapes the Rumen Microbiota to Improve the Ability of Tibetan Sheep (*Ovis aries*) To Adapt to the Cold Season. *Microbiol. Spectr. J.* **2023**, *11*, e02816-22. [CrossRef] [PubMed]
80. Ahmed, E.; Yano, R.; Fujimori, M.; Kand, D.; Hanada, M.; Nishida, T.; Fukuma, N. Impacts of Mootral on Methane Production, Rumen Fermentation, and Microbial Community in an in vitro Study. *Front. Vet. Sci.* **2021**, *7*, 623817. [CrossRef]
81. Stepanchenko, N.; Stefenoni, H.; Hennessy, M.; Nagaraju, I.; Wasson, D.E.; Cueva, S.F.; Räisänen, S.; Dechow, C.; Pitta, D.; Hristov, A. Microbial composition, rumen fermentation parameters, enteric methane emissions, and lactational performance of phenotypically high and low methane-emitting dairy cows. *J. Dairy Sci.* **2023**, *106*, 6146–6170. [CrossRef] [PubMed]
82. Pereira, A.M.; de Lurdes Nunes Enes Dapkevicius, M.; Borba, A.E.S. Alternative pathways for hydrogen sink originated from the ruminal fermentation of carbohydrates: Which microorganisms are involved in lowering methane emission? *Anim. Microbiome* **2022**, *4*, 5. [CrossRef] [PubMed]