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Article

Impact of an herbal additive on fermentation and rumen microbiota of lambs

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Abstract:

The use of phytobiotics has shown beneficial changes in animal production and health due to the presence of secondary metabolites, some of which have vitamin C activity due to the antioxidant function, important in ruminants. This research evaluated the effects of a phytobiotic made with *Phyllanthus emblica* and *Ocimum sanctum*, which has shown vitamin C activity, on ruminal fermentation and microbiota. Ten (10) male Hampshire × Suffolk lambs were housed individually and randomly assigned to two groups: 0 or 15 g/kg of dry matter of the herbal additive for 60 d. Ruminal fluid samples were collected to determine volatile fatty acid concentrations by gas chromatography, and microbiota analysis was performed through DNA extraction and 16S rRNA gene V3-V4 region sequencing. Alpha diversity was analyzed using Chao1, Shannon, and Simpson's reciprocal indices, while beta diversity was evaluated by principal coordinate analysis. Data were analyzed using an independent samples t-test and Pearson correlation. Supplementation decreased propionate concentration by 19.38 % (P= 0.05), with a tendency to increase the acetate:propionate ratio (P= 0.06), resulting in a 15.18 % increase in estimated methane concentration (P= 0.05), which affected fermentation efficiency. Regarding the microbiota, a 14.35 % decrease in Firmicutes (P= 0.06) and a 27.2 % increase in Bacteroidetes (P= 0.07) were observed. These results suggest that the phytobiotic modulates ruminal microbiota and fermentation.

Keywords: Lambs, Bacteroidetes, Phytobiotic, *Ocimum sanctum*, *Phyllanthus emblica*.

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Introduction

Ruminants have the capacity to synthesize vitamin C in liver and kidneys from glucose; therefore, it is not considered an essential nutrient; however, illness or stressful situation may reduce vitamin C levels[(1)]; and the ingredients commonly used for diets, such as hays, do not contain large amounts of vitamin C[(2)]; and also, current supplementation forms are also quickly degraded in the rumen[(3)]; therefore, alternatives have been sought to improve its availability, such as some phytobiotics that contain vitamin C activity and other secondary metabolites with antioxidant activity.

Phyllanthus emblica has been reported to have a high ascorbic acid content, while its main metabolites are gallic and ellagic acids; however, it also contains polyphenols, tannins, and alkaloids[4], some of the compounds have antimicrobial activity such as eugenol, thymol and caryophyllene[5].

The characterization of *Ocimum sanctum* reports that the metabolites present in this plant are mainly alkaloids, flavonoids, terpenoids, tannins, saponins, and phenols such as eugenol[6]; some of its compounds such as eugenol, ursolic acid, and caryophyllene have demonstrated antimicrobial activity[7]; however, the main metabolite is eugenol, which in addition to being antibacterial, also showed antifungal, antiviral, and antioxidant properties[8].

Previous research has reported that the addition of *P. emblica* and *O. sanctum,* in combination with other plants in a rumen fluid fermentation showed an increase in acetate production and a decrease in propionate and butyrate; leading in an increased acetate:propionate ratio[9]. In another experiment, supplementation increased volatile fatty acids (VFA) and butyrate production showing changes in microbiota[10]. Supplementation with a phytobiotic made with *P. emblica* and *O. sanctum* in fattening lambs improved carcass dressing and antioxidant capacity of the meat[3], and another research in twin-bearing ewes, supplementation increased the body weight of the fetuses and suggested that this type of antioxidants can be a good nutritional strategy for breeding ewes in adverse conditions[11]. Supplementation in dairy cattle reduced the incidence of mastitis and improved pregnancy rate[12].

Therefore, the objective of this study was to evaluate changes in ruminal fermentation and the ruminal microbiome in lambs fed with a feed plant additive based on *Ocimum sanctum* and *Phyllanthus emblica.*

Material and methods

Experimental design and diet

Ten (10) male Hampshire × Suffolk lambs were housed in individual pens (1.00 × 1.80 m) and assigned to a completely randomized design with two groups which consisted of dietary inclusion of a herbal additive at 0 or 15 g/kg dry matter (1.5 %) (Power-C, Nuproxa Mexico, Querétaro, México and Indian Herbs Research & Supply Co. Ltd.) fed for 60 d (n= 5 lambs/treatment) mixed into a basal ration for finishing lambs (Table 1). Dry matter intake was adjusted weekly according to lamb intake. Herbal additive is made with *Ocimum sanctum* and *Phyllanthus emblica* and contains 12 % hydrolysable tannins and 5.1 % gallic

acid quantified by HPTLC in certified laboratories[3] and 20 compounds with nutraceutical properties such as toluene, thymol, phenol, gamma-sitosterol, and other alcohols, aldehydes, and phenols[13].

Table 1: Composition of the basal ration of finishing lambs

Ingredients	**%**
Sorghum grain	54.44
Soybean meal	19.09
Alfalfa hay	10.00
Oat hay	10.00
Cane molasses	5.00
Mineral premix	1.00
Sodium chloride	0.30
Calcium carbonate	0.17
Chemical composition	**%**
Dry matter	88.65
Ether extract	2.52
Crude protein	17.4
Ash	6.31
Neutral detergent fiber	41.1
Acid detergent fiber	25.9

Ruminal fluid samples

After 60 d of feeding the average body weight of all animals was 45.77 ± 3.38 kg and ruminal fluid samples were obtained by an esophageal probe in the pre-prandial state and filtered through eight layers of gauze, this was separated in two samples, the first was separated into 1 mL aliquots, which were immediately placed on dry ice and stored in deep freezing (-80 °C) until analysis, for the second, 50 mL of ruminal fluid were used to measure the pH immediately with a pH-meter (Hanna Hi 2002-02, Rhode Island, USA) to later get acidified with metaphosphoric acid and frozen until VFA analysis.

VFA analysis

VFA were determined by gas chromatography using a Clarus 580 GC (Perkin Elmer, Waltham, MA, USA) with a HP-FFAP column 30 m x 0.25 mm, 0.25 µm (Agilent Technologies, Santa Clara, CA, USA), following the protocols described by Erwin *et al*[14] and modified by Miranda *et al*[15]. Molar proportions were calculated as the molar concentration of each individual VFA divides by the total VFA concentration, with the result multiplied by 100 to express it as a percentage. To estimate methane and carbon dioxide production, stoichiometric equations proposed by Wolin[16] and simplified by Van Soest[17] were applied. The following equations were used:

$$CO2 = (Acetate \div 2) + (Propionate \div 4) + (1.5 \times Butyrate)$$
$$CH4 = Acetate + (2 \times butyrate) - CO2$$

Ruminal fermentation efficiency was also estimated according to the methodology described previously[18].

Metagenomic analysis

Aliquots (250 µL) were taken from each sample, and metagenomic DNA extraction was performed with the ZymoBIOMICS® kit (Cat. No. D4300; Irvine, CA, USA) following the manufacturer's instructions. The quantification and quality of DNA obtained from the extraction process were evaluated with a NanoDrop 1000® (Thermo Fisher Scientific, Waltham, MA, USA); metagenomic DNA concentrations between 78.2 ng/µL and 129.8 ng/µL were obtained and 15 µL of each sample were sent to ZymoBiomics® (Zymo Research, Irvine, CA, USA) to be processed and analyzed by the targeted 16S rRNA V3-V4 sequencing service for microbiome analysis.

The bacterial 16S rRNA gene was sequenced using the Quick-16S™ NGS library preparation kit (Zymo Research, Irvine, CA, USA). The V3-V4 region of this gene was amplified with specific primers custom-designed by Zymo Research. The final pooled library was cleaned with Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA, USA), then quantified with TapeStation® (Agilent Technologies, Santa Clara, CA, USA) and Qubit® (Thermo Fisher Scientific, Waltham, MA, USA). As a positive control for each targeted library preparation, the ZymoBIOMICS® microbial community DNA standard (Zymo Research, Irvine, CA, USA) was used. Negative controls (i.e., extraction control blank and library preparation control blank) were included to assess the level of bioburden inherent in

the extraction process. The final library was sequenced on the Illumina® MiSeq™ platform with a v3 reagent kit (600 cycles). Sequencing was done with a 10% PhiX peak. Unique amplicon sequence variants were inferred from raw reads using DADA2 cleavage[19], which also served to eliminate chimeric sequences. The taxonomy assignment was carried out using Uclust from Qiime v.1.9.1[20] with Zymo's own database.

Statistical analysis

Data were analyzed using a completely randomized design with two groups (0 or 15 g/kg dry matter of the herbal additive). Alpha diversity and microbial richness were analyzed using Chao1, Shannon, and Simpson´s reciprocal indices. Additionally, Pearson correlation was also evaluated. Differences between groups were assessed using an independent samples t-test, considering $P<0.05$ as statistically significant. All statistical analyses were performed using the statistical analysis software SAS OnDemand for Academics[21].

For beta diversity a three-dimensional principal coordinate analysis (PCoA) plot was generated[22] using the matrix of paired-wise distance between samples calculated by the Bray-Curtis dissimilarity using unique amplicon sequence variants (ASV)[23]. Composition alpha, and beta diversity visualization analyses were also performed with QIIME v.1.9.1.

Results

Rumen microbiota and fermentation pattern

Supplementation with the herbal additive decreased propionate concentration ($P= 0.05$), showed a tendency to increase acetate:propionate ratio ($P= 0.06$) and a negative correlation was detected between acetate and propionate concentration (r= -0.774, $P<0.01$); in regard to relative abundance of phyla, Firmicutes decreased and Bacteroidetes showed a tendency to increase ($P= 0.07$) also, a negative correlation was detected between both phyla (r= -0.904, $P<0.001$). Estimated molar methane concentration was increased ($P= 0.05$). Although no statistically significant difference was observed in total VFA, supplementation resulted in a 14.6 % increase (Table 2).

Table 2: Effect of herbal additive on ruminal fermentation and VFA concentration in lambs

	C Powder® (g/kg DM)		SEM	CV (%)	*P*-value
	0	15			
DM intake, kg/d	1.599	1.592	0.066	9.66	0.94
Total VFA, mM	67.71	82.31	6.477	27.31	0.28
pH	6.81	6.84	0.074	3.41	0.81
Acetate, %	56.47	61.70	1.668	8.93	0.12
Propionate, %	30.13	24.59	1.477	17.07	0.05
Butyrate, %	13.38	13.70	1. 072	25.02	0.89
Acetate:propionate ratio	1.93:1	2.54:1	0.168	23.73	0.06
CO_2, % molar	55.85	57.56	1.193	6.65	0.51
CH_4, % molar	27.40	31.56	1.108	11.88	0.05
Fermentation efficiency	84.81	82.48	0.622	2.35	0.05

DM= dry matter; SEM= standard error of the mean; CV= coefficient of variation; VFA= volatile fatty acids.

At the phylum level, additive supplementation tended to increase the relative abundance of Bacteroidetes and reduce the abundance of Firmicutes ($P<0.10$) compared to the control, also tended to reduce the abundance of Corynebacteriaceae ($P= 0.06$), and reduces Lachnospiraceae, Rikenellaceae, Defluviitaleaceae, and Eubacteriacea ($P\leq0.05$) compared to the unsupplemented lambs (Table 3). Some bacterial families were detected with the additive supplementation (Staphylococcaceae, Carnobacteriaceae, Rhodospirillaceae, and Anaeroplasmataceae), and although there are no statistical differences, their presence biologically should be considered.

Table 3: Effect of herbal additive on the Firmicutes:Bacteroidetes ratio and relative abundance of the main phyla and families of ruminal microbiota

	C Powder®(g/kg DM)		SEM	CV (%)	*P*-value
	0	15			
Bacteroidetes	25.00	31.80	1.890	21.04	0.07
Firmicutes	62.16	53.24	2.454	13.45	0.06
Firmicutes:Bacteroidetes ratio	2.66:1	1.70:1	0.263	38.15	0.06
Lachnospiraceae	32.91	23.70	2.303	26.59	0.03
Prevotellaceae	23.70	27.62	1.521	19.80	0.21
Veillonellaceae	12.30	12.80	1.647	43.61	0.88
Ruminococcaceae	9.41	10.66	0.907	30.14	0.52
Succinivibrionaceae	5.98	5.18	1.116	65.89	0.74
Methanobacteriaceae	2.21	5.08	0.964	90.95	0.14
Bifidobacteriaceae	0.31	0.40	0.049	46.11	0.42
Corynebacteriaceae	3.0	1.96	0.285	37.45	0.06
Bacteroidaceae	1.35	2.72	0.458	77.14	0.14
Rikenellaceae	0.53	1.58	0.211	72.68	0.01
Anaerolineaceae	0.0	0.02	0.009	331.66	0.29
Fibrobacteraceae	0.13	0.24	0.046	84.55	0.27
Staphylococcaceae	0.0	0.04	0.021	222.48	0.10
Carnobacteriaceae	0.0	0.02	0.009	331.66	0.29
Christensenellaceae	0.63	1.66	0.345	104.20	0.14
Defluviitaleaceae	0.15	0.06	0.021	64.22	0.02
Eubacteriaceae	0.21	0.10	0.030	62.75	0.05
Family XIII	1.53	1.24	0.121	28.74	0.24
Other Clostridials	0.05	0.02	0.027	254.21	0.61
Erysipelotrichaceae	3.30	2.00	0.722	88.48	0.39
Acidaminococcaceae	0.86	0.94	0.175	64.78	0.84
Synergistaceae	0.05	0.56	0.214	252.81	0.25
Spirochaetaceae	0.03	0.24	0.038	99.94	0.34
Desulfovibrionaceae	0.15	0.14	0.015	35.90	0.76
Rhodospirillaceae	0.00	0.02	0.009	331.66	0.29
Anaeroplasmataceae	0.00	0.02	0.009	331.66	0.29
Cardiobacteriaceae	0.01	0.00	0.009	331.66	0.38
Saccharibacteria	0.24	0.13	0.053	97.82	0.34
Other	0.90	0.88	0.133	49.80	0.94

DM= dry matter; SEM= standard error of the mean; CV= coefficient of variation

Rumen microbiota structure and composition

A total of 465,134 raw sequences were obtained from the V3-V4 region of 16S rRNA gene analysis, within a range of 42,050 to 51,082 (average 46,513 per sample). After quality control analysis, as well as chimera detection and elimination (194,402 sequences), the total sequences analyzed were 189,576, in a range of 16,788 to 21,472 (average 18,958 per sample). The microbial communities of the rumen from lambs fed with the additive showed similar values of richness (Chao1), homogeneity (Shannon), and diversity (Simpson reciprocal) indices (Table 4) without differences among treatments ($P>0.05$).

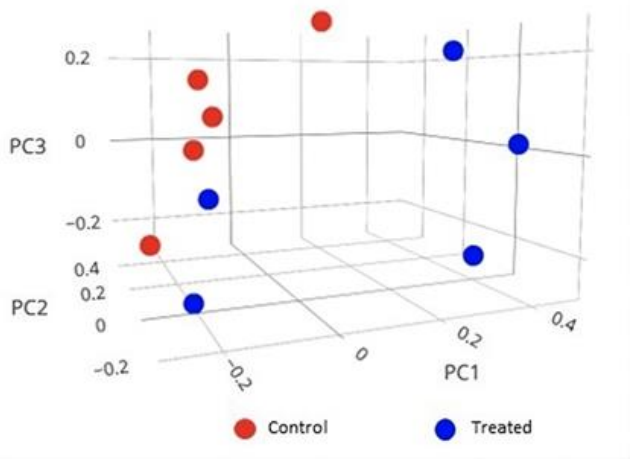
Table 4: Effect of herbal additive addition on rumen microbial diversity in lambs

Estimator/Indices	C Powder® g/kg DM		SEM	CV (%)	*P*-value
	0	15			
Chao1	211.80	267.00	20.71	27.35	0.20
Shannon	6.66	6.95	0.15	6.85	0.35
Simpson reciprocal	55.45	68.84	7.84	39.92	0.43

DM= dry matter; SEM= standard error of the mean; CV= coefficient of variation.

Beta diversity is a measure of differences in microbial diversity between samples. The composition profiles of the samples showed differences between control animals and those fed with the herbal additive since similar profiles of control samples are closer to each other in the plot. Samples from treated animals, although dispersed from each other, form a different group to the control, suggesting that the additive has a modeling effect on the rumen microbiota of supplemented lambs (Figure 1).

Figure 1: Beta diversity analysis of ruminal microbial communities in unsupplemented lambs (red dots) and lambs supplemented with the herbal additive (blue dots)



Discussion

Based on the results obtained from the microbiota analysis using V3-V4 region sequencing, no significant changes were observed in alpha diversity indices, suggesting that supplementation did not alter microbial richness, homogeneity, or diversity. Regarding beta diversity, the PCoA plot showed a dispersion of points, and samples from the supplemented group; although separated from each other, formed a distinct cluster from the control group; this indicates that supplementation caused subtle changes in the microbial community composition. These modifications were reflected in the relative abundance of certain phyla and families, which influenced the observed fermentation patterns. This is similar to other research where no changes in alpha diversity were observed; however, in the PCoA the points formed two different groups, suggesting alterations in the ruminal bacterial diversity[24].

The phyla Bacteroidetes and Firmicutes are the main mediators in the generation of hydrolysis and VFA production, and the proportion of these affects the fermentation pattern and metabolism of ruminants[25,26,27]. Herbal additive supplementation reduced the abundance of the Firmicutes phylum, which includes Gram-positive bacteria responsible for the breakdown of exogenous peptides and amino acids[28]. Previous research in cattle showed that this phylum increases when there are high amounts of concentrate in the diet[29]. On the other hand, Bacteroidetes phylum is more efficient in the decomposition of cellulose, hemicellulose and pectins due to the enzyme glycoside hydrolase and polysaccharide lyase[28]; therefore, this phylum has been decreased in high grain diets[30], in contrast to a greater proportion with forage-rich diets[29]. In this trial, Firmicutes:Bacteroidetes ratio decreased, which could indicate that ruminal fermentation was favored for cell walls digestion.

Some families of Firmicutes phylum were decreased, such as Lachnospiraceae, Defluviitaleaceae, and Eubacteriaceae. Bacteria of Lachnospiraceae family have the ability to produce the enzyme cellobiose phosphorylase[31]. Within the Bacteroidetes phylum, there are reports that Prevotellaceae family plays an important role in the utilization of polysaccharides from plants such as hemicellulose, xylans, pectins, and starch to generate carboxylates and subsequently form VFA, also has the capacity to increase its population in acidic environments[32,33]. Previous research mentions that this family is the most abundant when the diet is high in fiber and is associated with an increase in the production of total VFA and acetate[29,34]. In this study, Prevotellaceae family was higher by phytobiotic supplementation which could indicate that supplementation favors the microbiota to improve cell walls digestion. Other families of this phylum have the ability to ferment different types of carbohydrates and form succinic, acetic, and formic acid[25,35]. Among the changes observed was the reduction of Corynebacteriaceae family, which contains some bacteria that

participate in the incidence of opportunistic infections[36] due to secondary metabolites present in the phytobiotic.

The decrease in propionate concentration, along with the trend toward an increased acetate:propionate ratio, suggests a shift in fermentation favoring acetate concentration. This change is consistent with the observed increase in Rikenellaceae family, which is negatively associated with propionate production[33,34,37]. This could explain the observed decrease in propionate concentration, in supplemented lambs; in this sense, the negative correlation between propionate and acetate indicates greater acetate concentration, which may result in a higher availability of carbon and hydrogen for methane synthesis. Acetate formation releases hydrogen, which is a key substrate for methanogenesis, whereas propionate formation consumes hydrogen[29]. Therefore, this fermentation pattern could be associated with a potential increase in methane synthesis. Although no significant differences were observed in the abundance of methanogenic archaea, the estimated methane concentration increased, which along with the decrease in propionate and the change in the acetate:propionate ratio indicate a possible increase in hydrogen production, causing a decrease in fermentation efficiency[37].

Some researchers report that the use of some phytobiotics helps to reduce methane production[38], this should not be generalized about their action at the ruminal level. This study shows that the effects caused by these products are not always favorable. On the other hand, there are reports showing that some secondary compounds decrease ruminal methanogenesis; however, the effect cannot be attributed only to the direct effect on methanogenesis, but also on protozoa and an increase in acetate production[39].

Although no significant differences were found in total VFA concentration, the numerical increase observed should be considered, because VFA are the main energy source for ruminants. An increase in their concentration could reduce ruminal pH, favoring VFA protonation and facilitating their passive absorption through the rumen epithelium, which could have a positive impact on energy availability[40,41].

The evaluation of these additives requires further research to be able to observe the effects that supplementation can have on animals and not only on ruminal fermentation due to the fact that supplementation with other herbal additives has shown positive effects on animal production; in dairy calves, through a gene expression analysis, an improvement in lipids, carbohydrates and proteins metabolism were detected, in addition to improving the immune response[42].

Conclusions and implications

Dietary inclusion of herbal additive made with *Ocimum sanctum* and *Phyllanthus emblica* at 15 g/kg of dry matter in lambs modified the ruminal microbiota by increasing bacteria from the Bacteroidetes phylum. Additionally, an increase in the bacterial family Rikenellaceae was observed, which is associated with a decrease in propionate production; this may be related to the lower propionate concentration in supplemented lambs. On the other hand, the decrease in propionate may favored methane production, which led to an increase in the estimated methane concentration in supplemented lambs, affecting fermentation efficiency.

Acknowledgements and conflict of interest

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Literature cited:

1. Akinmoladun OF. Stress amelioration potential of vitamin C in ruminants: a review. Trop Anim Health Prod 2022;54(1):24.

2. Escobar-Ramírez F, Espinoza-Ochoa T, Hinojosa-Benavides RA, De la Cruz-Marcos RN. Sustitución parcial y total de alfalfa fresca por heno en la alimentación de cuyes (*Cavia porcellus*) en crecimiento y engorde: una alternativa para la época de estiaje. J Selva Andina Anim Sci 2023;10(1):16-29.

3. Lozano-Sánchez M, Mendoza-Martínez GD, Martínez-García JA, Torre-Hernández ME, Chamorro-Ramírez FH, Martínez-Aispuro JA, *et al*. Evaluation of polyherbal with vitamin C activity on lamb performance and meat characteristics. Rev Bras Zootec 2021;50:e20200166.

4. Jhaumeer Laulloo S, Bhowon MG, Chua LS, Gaungoo H. Phytochemical screening and antioxidant properties of *Phyllanthus emblica* from mauritius. Chem Nat Compd 2018; 4(1):50-55.

5. Begum MA, Hossain R, Jain D, Murti Y, Agrawal KK, Janmeda P, *et al*. Recent insights into the antimicrobial properties of *Phyllanthus emblica* L.: a comprehensive review of wonder berry. Chem Biodivers 2024;21(9):e202400747.

6. Borah R, Biswas SP. Tulsi (*Ocimum sanctum*), excellent source of phytochemicals. Int J Environ Agric Biotechnol 2018;3(5):1732-1738.

7. Panchal P, Parvez N. Phytochemical analysis of medicinal herb (*Ocimum sanctum*). Int J Nanomater Nanotechnol Nanomed 2019;5(2):008-011.

8. Nisar MF, Khadim M, Rafiq M, Chen J, Yang Y, Wan CC. Pharmacological properties and health benefits of eugenol: a comprehensive review. Oxid Med Cell Longev 2021;2021:2497354.

9. Chaturvedi I, Dutta TK, Singh PK, Sharma A. Effect of combined herbal feed additives on methane, total gas production and rumen fermentation. Bioinformation 2015;11(5):261-266.

10. Chaturvedi I, Dutta TK, Singh PK, Chatterjee A, Mandal DK, Bhakat C, Mohammad A, Das AK. Effect of supplementation of phytogenic feed additives on intake, *in vitro* fermentation, growth performance and carcass traits in weaned Barbari kids reared under intensive feeding. Trop Anim Health Prod 2022;54(2):150.

11. Parraguez VH, Sales F, Peralta OA, De Los Reyes M, Campos A, González J, Peralta W, Cabezón C, González-Bulnes A. Maternal supplementation with herbal antioxidants during pregnancy in swine. Antioxidants 2021;10(5):658.

12. Mendoza-Martinez GD, Ortega-Alvarez NI, Hernández-García PA, Espinosa-Ayala E, Díaz-Galván C, Frutis-Moto K, *et al*. Impact of a plant feed additive containing vitamin C on Holstein cow's performance under heat stress conditions. Emir J Food Agric 2022;34(3):248-259.

13. Villanueva M, Espinosa-Reyes G, Flores-Ramirez R, Rojas-Velazquez AN, López JCG, Vazquez-Valladolid A, *et al*. Herbal vitamin C prevents DNA oxidation and modifies the metabolomic water profile of tilapia (*Oreochromis* spp.). Life 2022;12:1243.

14. Erwin ES, Marco GJ, Emery EM. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. J Dairy Sci 1961;44(9):1768-1771.

15. Miranda LA, Lee-Rangel HA, Mendoza-Martínez GD, Crosby-Galván MM, Relling AE, Pinos-Rodríguez JM, *et al*. Influence of calcium propionate on *in vitro* fermentation of sorghum-based diets. Rev Fac Cienc Agrar 2017;49(1):185-192.

16. Wolin MJ. A theoretical rumen fermentation balance. J Dairy Sci 1960;43(10):1452-1459.

17. Van Soest PJ. Nutritional ecology of the ruminant. 2nd ed. Cornell University Press; 1994.

18. Mendoza MGD, Hernandez-Garcia PA, Plata PFX. Nutrición animal cuantitativa. Primera ed. Ciudad de México: Universidad Autónoma Metropolitana; 2022.

19. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13(7):581-583.

20. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al*. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7(5):335-336.

21. SAS Institute. Statistical Analysis System (SAS On Demand for Academics). 2021.

22. Vázquez-Baeza Y, Pirrung M, González A, Knight R. EMPeror: a tool for visualizing high-throughput microbial community data. GigaScience 2013;2(1):16.

23. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr 1957;27(4):325-349.

24. Wang L, Wu D, Yan T, Wang L. The impact of rumen cannulation on the microbial community of goat rumens as measured using 16S rRNA high-throughput sequencing. J Anim Physiol Anim Nutr 2018;102(1):175-183.

25. Jami E, White BA, Mizrahi I. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PLoS ONE 2014;9(1):e85423.

26. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027-1031.

27. Yang Y, Wang M, Yan S, Yong X, Zhang X, Awathi MK, Xi Y, Zhou J. Effects of hydrochar and biogas slurry reflux on methane production by mixed anaerobic digestion of cow manure and corn straw. Chemosphere 2023;310:136876.

28. Cholewińska P, Wołoszyńska M, Michalak M, Czyż K, Rant W, Smoliński J, Wyrostek A, Wojnarowski K. Influence of selected factors on the Firmicutes, Bacteroidetes phyla and the Lactobacillaceae family in the digestive tract of sheep. Sci Rep 2021;11(1):23801.

29. Wang R, He S, Huang D, Wu D, Peng H, He S, *et al*. The response of rumen pH, fermentation parameters and rumen bacteria to feeds of different concentrate to roughage ratios in buffalos. Front Microbiomes 2022;1:1053794.

30. Chen H, Wang C, Huasai S, Chen A. Effects of dietary forage to concentrate ratio on nutrient digestibility, ruminal fermentation and rumen bacterial composition in Angus cows. Sci Rep 2021;11(1):17023.

31. Ricci S, Pacífico C, Castillo-Lopez E, Rivera-Chacon R, Schwartz-Zimmermann HE, Reisinger N, Berthiller F, Zebeli Q, Petri RM. Progressive microbial adaptation of the bovine rumen and hindgut in response to a step-wise increase in dietary starch and the influence of phytogenic supplementation. Front Microbiol 2022;13:920427.

32. Liang J, Zheng W, Zhang H, Zhang P, Cai Y, Wang Q, Zhou Z, Ding Y. Transformation of bacterial community structure in rumen liquid anaerobic digestion of rice straw. Environ Pollut 2021;269:116130.

33. Zened A, Combes S, Cauquil L, Mariette J, Klopp C, Bouchez O, Troegeler-Meynadier A, Enjalbert F. Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. FEMS Microbiol Ecol 2013;83(2):504-514.

34. Zhu X, Liu B, Xiao J, Guo M, Zhao S, Hu M, Cui Y, Li D, Wang C, Ma S, Shi Y. Effects of different roughage diets on fattening performance, meat quality, fatty acid composition, and rumen microbe in steers. Front Nutr 2022;9:885069.

35. Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol 2008;6(2):121-131.

36. Zhi XY, Jiang Z, Yang LL, Huang Y. The underlying mechanisms of genetic innovation and speciation in the family Corynebacteriaceae: A phylogenomics approach. Mol Phylogenet Evol 2017;107:246-255.

37. Xia G, Sun J, Fan Y, Zhao F, Ahmed G, Jin Y, Zhang Y, Wang H. β-Sitosterol attenuates high grain diet-induced inflammatory stress and modifies rumen fermentation and microbiota in sheep. Animals 2020;10(1):171.

38. Alayón-Gamboa JA, Albores-Moreno S, Jiménez-Ferrer G, Alarcón-Zúñiga B, Miranda-Romero LA, Pérez-Luna EJ, Canul-Solís J. Tropical tree foliage supplementation in ruminants improves rumen fermentation and the bacterial profile and decreases methane production. Anim Biotechnol 2023;34(9):4510-4522.

39. Ugbogu EA, Elghandour MMMY, Ikpeazu VO, Buendía GR, Molina OM, Arunsi UO, Emmanuel O, Salem AZM. The potential impacts of dietary plant natural products on the sustainable mitigation of methane emission from livestock farming. J Clean Prod 2019;213(10):915-925.

40. Yohe TT, Schramm H, White RR, Hanigan MD, Parsons CLM, Tucker HLM, Enger BD, Hardy NR, Daniels KM. Form of calf diet and the rumen. II: Impact on volatile fatty acid absorption. J Dairy Sci 2019;102(9):8502-8512.

41. Millen DD, Pacheco RDL, Da Silva Cabral L, Cursino LL, Watanabe DHM, Rigueiro ALN. Ruminal acidosis. In Millen DD, *et al*, editors. Rumenology. Switzerland: Springer International Publishing; 2016:127-156.

42. Díaz-Galván C, Méndez-Olvera ET, Martínez-Gómez D, Gloria-Trujillo A, Hernández-García PA, Espinosa-Ayala E, *et al*. Influence of a polyherbal mixture in dairy calves: growth performance and gene expression. Front Vet Sci 2021;7:623710.