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Article

Potential of a phytobiotic based on *Acacia concinna* and the red seaweed *Palmaria palmata* to reduce *in vitro* ruminal methane production

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Abstract:

Changes in methane and carbon dioxide production and their effects on the ruminal microbiota were evaluated *in vitro* by incubating alfalfa with an herbal additive formulated with *Acacia concinn*a and with a supplement containing red seaweed *Palmaria palmata*. The metabolites of the supplement with *Palmaria palmata* were characterized, and 20 chemical compounds were found, of which phenols, terpenes, halogenated compounds, and alkanes stood out. The inclusion of the herbal additive reduced (P<0.01) ruminal methane by 29 % and red seaweed by 56 %. The volatile fatty acid (VFA) molar concentration increased (P<0.05) with the seaweed additive. The two additives increased the proportions of acetate and propionate and reduced that of butyrate (P<0.05) compared to the control. No differences (P>0.05) were detected in the abundance of most microbial families, only minor changes in *Rikenellaceae*; just *Cellulomonadaceae* increased significantly (P>0.001) with the herbal additive. The two additives showed methane reductive potential, which was more pronounced for *Palmaria palmata*.

Keywords: Gas production, Methanogenesis, Natural additive, VFA.

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Introduction

Against the background of a changing climate, it is crucial to reduce ruminal methane emissions, in addition to the fact that methane emission represents an energy loss for ruminants[(1)]. Among the natural compounds that can reduce methane are plant saponins[(2)], available in some polyherbal mixtures[(3)], and the halogenated compounds synthesized by some seaweed species[(4,5)].

A previously described herbal additive based on *Acacia concinna* has a standardized content of saponins and shows biological effects similar to those of coccidiostats in broilers[(3)]. It affects the productive performance of feedlot lambs[(6)] and dairy calves[(7)] and also contains other secondary metabolites, highlighting 43 organic compounds such as thymol, cinnamon, caryophyllene, phenols, oleic acid, polyunsaturated fatty acids, and vitamin E[(7)].

Saponins have toxic effects on protozoa but also form complexes with the lipid membrane of bacteria, affecting some bacterial populations(2). The inclusion of saponins at low dietary concentrations can be beneficial for ruminants, improving ruminal fermentation efficiency by decreasing methanogenesis, which is associated to the saponin effects on *Archaea* and rumen protozoa(8-11).

Some species of the red macroalgae genus *Asparagopsis* produce metabolites with anti-methanogenic effects(12-16), and there is evidence that *Asparagopsis taxiformis* can significantly reduce methane emissions in sheep(17) and beef cattle(18,19). However, other algae species should be evaluated in terms of their methane reduction potential based on their biochemical characteristics(20,21). When comparing different seaweed species in *in vitro* ruminal assays, *Palmaria palmata* showed higher digestibility and gas production as well as volatile fatty acid (VFA) and propionate concentrations than other species and produced lower ruminal methane concentrations(22). *Palmaria palmata* is a red alga (Rhodophyta) formerly called *Rhodymenia palmata* (Linnaeus) and belongs to the family *Palmariaceae*(23). It has been subjected to different forms of cultivation(24) and is marketed as a supplement for human consumption, making it available as a feed additive for ruminants. In this context, the objective of this study was to compare the *in vitro* methane reduction potential of a feed plant additive made from *Acacia concinna* versus a product made from *Palmaria palmata*, evaluating the changes in methane production, ruminal fermentation and effects on the rumen microbiota.

Material and methods

Animals and inoculum

The ruminal inoculum was collected by a trained veterinarian from crossed lambs with an esophageal probe in preprandial conditions in order to reduce variation(25). The procedures were approved by the Animal Welfare Committee of the UAEM Amecameca University Center of the Autonomous University of the State of Mexico (Protocol #0201-2023). The donors of ruminal liquid were fed a diet with 75 % corn silage and 25 % commercial concentrate with 12 % crude protein. This fluid used as inoculum, was placed in a thermo at 39 ºC and transported to the nutrition laboratory, where it was filtered through four layers of cheese cloth and saturated with carbon dioxide.

Additives evaluated

Two commercial products were tested: the herbal product formulated with *Acacia concinna* (Peptasan®, Indian Herbs Specialties Pvt and Nuproxa Mexico) and the red seaweed capsules (Solaray Inc; Salt Lake City, UT) elaborated with *Palmaria palmata*, which can be obtained via the internet as supplement for human consumption.

Product characterization

Samples of the red seaweed *Palmaria palmata* were used to characterize the secondary metabolites using a gas chromatograph coupled to a mass spectrometer (GC-MS). Extraction was performed using an ultrasonic processor (GEX130, 115 V 50/60 Hz) equipped with a 3-mm titanium tip and mechanical stirrers (Cole-Parmer, IL, USA) as described by Roque-Jiménez *et al*(26). One gram of red seaweed product was mixed with 10 mL of hexane to separate the organic phase by an ultrasonic processor with a 70 % amplitude and centrifuged at 3,500 rpm for 5 min, followed by concentration in a water bath with a direct CO_2 probe for 5 min at 37 °C to recover 1 mL of the extracted mixture (Zymark, Turbovap LV Concentration Evapotarot, NB, USA). The metabolites were characterized by a gas chromatograph (GC-HP 6890) coupled to a mass spectrophotometer (MSHP 5973), using a capillary column (60 m in length, 0.255 mm in diameter, 0.25 µm in thickness) (HP 5MS, Agilent) under conditions described by Roque-Jiménez *et al*(26). The herbal product was previously characterized by Lee *et al*(7) and saponins content reported by Sánchez-Hernández *et al*(3).

In vitro gas production and rumen fermentation

Alfalfa was incubated as a main substrate with 0 % and 2 % red seaweed or herbal additive, using five 120 ml amber flasks for each treatment and their controls; two runs were conducted. Each flask contained 0.5 g of alfalfa ground to a particle size of 2 mm and 90 mL of ruminal fluid (added to each flask with a continuous flow of CO_2) and was sealed. Five flasks were included as blanks containing only ruminal inoculum. The flasks were placed in a water bath at 39 ºC, and the gas pressure was recorded at 3, 6, 9, 12, 24, 36, and 48 h of incubation using a manual manometer (Amphenol SSI Technologies)(27). To estimate CH_4 and CO_2, it was used the methodology proposed by Menke and Steingass(28), recording the gas volume accumulated up to 48 h with a 60-mL graduated hypodermic syringe. In each measurement, the gas trapped in the syringe was transferred by injection to another hermetically closed flask, which contained 40 mL of a sodium hydroxide solution (1M KOH) prepared with distilled water, mixing the gas to fix the carbon dioxide to form potassium bicarbonate[29,30]. The residual volume was used to estimate methane[31].

Flasks were filtered at the final incubation time to estimate the percentage of dry matter disappearance. A subsample of ruminal fluid at 24 h of incubation was acidified with metaphosphoric and frozen until the analysis of VFA by gas chromatography[32], using a Perkin Elmer Clarus 580 with a capillary column of 30 m x 0.25 mm x 25 µm (Agilent Technologies model HP-FFAP) and nitrogen as carrier gas.

DNA isolation and metagenomic analyses

Ruminal fluid from six tubes at 12 h of fermentation were cryopreserved and stored immediately at -80 °C until DNA extraction; 2 mL of each sample were mixed with 6 mL of DNA/RNA Shield (Zymo Research, Cat. No.: R1100-250; Lot. No.: 219429) and sent to Zymo Research (Irvine, CA) for metagenomic-DNA extraction, quantification, sequencing and analysis by the targeted sequencing service for microbiome, as described by Mendoza-Martínez *et al*[33].

Sequencing of the microbial 16S rRNA gene was performed using the Quick-16S™ NGS library preparation kit (Zymo Research, Irvine, CA). 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) and subsequently quantified with TapeStation™ (Agilent Technologies, Santa Clara, CA) and Qubit™ (Thermo Fisher Scientific, Waltham, WA). As a positive control for each targeted library preparation, the ZymoBIOMICS™ microbial community DNA standard (Zymo Research, Irvine, CA) was employed. 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 pipeline[34], which also served to eliminate chimeric sequences. The taxonomy assignment was carried out using Uclust from Qiime v. 1.9.1[35] with Zymo's own database. Composition as well as alpha and beta diversity analyses were also performed with Qiime v. 1.9.1.

Statistical analysis

Data normality was tested with the Shapiro Wilk test, and the results were analyzed in a completely randomized block design[36] using run as a blocking criterion with the following model:

$Y_i = \mu + B_i + T_j + B_i * T_j + e_{ij}$

Where:

Y is the dependent variable,

$\boldsymbol{\mu}$ is the overall population of the mean,

$\mathbf{B_i}$ is the fix effect of run (Block),

$\mathbf{T_i}$ is the fix effect of treatment,

eij is the residual error, using the Block x treatment interactions a an experimental error[37].

When significant effects were detected, and means were compared by orthogonal contrasts. The *in vitro* gas production kinetics parameters (lag phase (h), maximum volume (Vm), and gas production rate (S)) were estimated with the model Vo= Vm / (1 + e (2-4 * s * (tL))), proposed by Menke and Steingass[28] and parameters were estimated by non-linear regression[38]. Software used was SAS® OnDemand for Academics (https://www.sas.com/en_us/software/on-demand-for-academics.html).

Results

Product characterization

Gas chromatography coupled with mass spectrophotometry revealed 20 chemical compounds present in *Palmaria palmata* (Table 1), including a variety of phenols, terpenes, and alkanes such as heptadecane, followed by glycerol tricaprylate, which is a triglyceride of octanoic acid esters and other components such as 1,2-benzenedicarboxylic acid and dioctyl ester.

Table 1: Identification of volatile compounds in the red seaweed *Palmaria palmata* supplement using gas chromatograph coupled to a mass spectrometer

Retention time (m)	Area Pct	Compound	Group
22.49	41.6478	Heptadecane	Alkane
40.64	13.556	Glycerol tricaprylate	Octanoic acid esters triglyceride
35.74	9.7744	1,2-Benzenedicarboxylic acid, dioctyl ester	Dicarboxylic derivative of benzene
20.22	6.1088	2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	Terpene
39.75	3.5267	Silane, 1,4-Bis(trimethylsilyl)benzene	Aromatic silica compound
24.5	3.4513	2-pentadecanone, 6,10,14-trimethyl-	Sesquiterpene
28.48	3.1178	Phytol	Acyclic natural
28.51	2.6536	Phytol	diterpene alcohol, constituent of chlorophyll
18.01	2.4876	Benzenemethanol, alpha,4-dimethyl-, (+/-.)-	NAI
24.41	1.8725	Bicyclo (3.1.1) heptane, 2,6,6-trimethyl-, (1R-(1-alpha,2-beta,5-alpha))-	Terpenoid
39.98	1.655	1H-pyrrole-2,5-dione, 1-(4-chlorophenyl)-	NAI
36.61	1.5279	2(3H)-furanone, dihydro-5-tetradecyl-	Furan
19.75	1.4862	Phosphine, (1,1-dimethylethyl) methylphenyl-	NAI
40.73	1.2167	2-ethylacridine	NAI
19.07	1.2149	Phenol, 2,4-bis(1-methylpropyl)-	Phenol
19.14	1.0861	3-buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo (4,1,0) hept-1-yl)-	NAI
25	1.0368	3,7,11,15-tetramethyl-2-hexadecen-1-ol	NAI
25.58	0.9026	Pentadecanoic acid, 14-methyl-, methyl ester	Fatty acid
39.95	0.855	1,3-bis(trimethylsilyl)benzene	NAI
40.01	0.8225	2,4-cyclohexadien-1-one, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-	Ketone compound

NAI= Not available information.

In vitro gas production and rumen fermentation

In vitro gas production (Table 2) was reduced with the addition of the herbal product and red seaweed by 2 % and 7 %, respectively, compared to that of the control group. The gas production rate was slower with red seaweed (*P*<0.01), whereas the lag phase and the *in vitro* dry matter (DM) digestibility were not affected. Methane was reduced (*P*<0.01) with the herbal additive by 29 % and by 56 % with the red seaweed in comparison with that of the controls; however, the CO_2 level was not affected. The molar VFA concentration increased (*P*<0.05) with the red seaweed additive, which showed greater proportion of acetate and reduced propionate and butyrate levels (*P*<0.01) compared to those of the control group. The herbal product results in increased proportions of acetate and reduced propionate levels (*P*<0.05).

Table 2: Effects of a phytobiotic based on *Acacia concinna* and a commercial supplement containing red seaweed *Palmaria palmata* on kinetics of *in vitro* gas production, ruminal fermentation and methane

	Control	Red seaweed	Herbal additive[x]		Contrast *P*-value	
		Palmaria palmata	***Acacia concinna***	**SEM**	**Control *vs* seaweed**	**Control *vs* herbal additive[x]**
V max, mL/g	168.16	156.76	165.03	2.360	0.014	0.048
S, %/h	0.039	0.031	0.037	0.002	0.018	0.044
Lag, h	1.94	0.63	1.57	0.588	0.164	0.301
IVDMD %	69.60	69.42	68.57	0.951	0.896	0.553
VFA total mmol/L	84.63	87.57	86.22	0.761	0.035	0.256
Acetic acid, %	60.64	77.77	74.67	0.769	0.0001	0.029
Propionic acid, %	23.41	14.18	17.74	0.664	0.0001	0.009
Butyric acid, %	16.36	7.41	7.72	0.751	0.0002	0.780
CH_4, mL	26.00	11.50	18.50	1.179	0.0001	0.006
CO_2, mL	59.00	59.00	58.00	1.724	0.998	0.696

V max= maximum volume of gas; S= rate of gas production; Lag= lag phase; IVDMD= *in vitro* dry matter digestibility; SEM= standard error of the mean. [x]Peptasan® herbal additive.

Metagenomic analyses

From the 6 samples, were obtained 2’630,796 raw sequences in total from the V3–V4 region of the 16S rRNA gene, within a range of 208,618 to 571,150 (average 438,466 sequences per sample). After performing quality filtering, as well as chimera detection and elimination (117,294 sequences), the total unique amplicon sequence variants (ASV) analyzed were 874,117, in a range of 67,716 to 213,108 (average 148,216 per sample). Shannon (homogeneity) and Simpson reciprocal (diversity) indices, as well as Chao1 richness estimator, were calculated for each treatment (Table 3), having been normalized to 20,000 sequences.

Table 3: Effects of a phytobiotic based on *Acacia concinna* and a commercial supplement containing red seaweed *Palmaria palmata* on ruminal microbial diversity

	Control	**Red seaweed**	**Herbal additive**^{X}	**SEM**	***P*-value**
Chao1	804.917	857.677	495.619	67.721	0.0595
Shannon	4.3405	4.8785	4.417	0.830	0.874
Simpson reciprocal	3.971	5.008	5.018	1.063	0.750

SEM= standard error of the mean. ^{X}: Peptasan® herbal additive.

Analysis of alpha diversity indexes of the microbial communities of the rumen did not show significant differences between treatments. However, the tendency to decrease species richness was detected in the presence of the polyherbal additive (Table 3, Chao1), probably due to the presence of diverse plant secondary metabolites with antimicrobial activity, as will be discussed further.

Twenty-one (21) bacterial/archaeal families with a relative abundance >0.1 % were identified in any of the samples (Table 4); families with lower abundances were grouped into "others". No significant differences (*P*>0.05) were detected for most of the microbial families. The abundance of methanogenic *Archaea* was not affected with the herbal product, but apparently increased by 50 % with the red seaweed. The abundance of *Rikenellaceae* was reduced by 8 % with red seaweed and by 36 % with the herbal additive, as well as *Micrococcaceae*, which apparently disappear with red seaweed, and was reduced 93 % with herbal additive. *Microbacteriaceae* family could only be detected in the treatments with the herbal additive, which was also the case for *Corynebacteriaceae, and Sanguibacteraceae*, albeit without a statistically significant difference. In turn, *Cellulomonadaceae* was increased by 650 % with red algae and 9,000 % (*P*>0.001) with the additive.

Table 4: Effects of a phytobiotic based on *Acacia concinna* and a commercial supplement containing red seaweed *Palmaria palmata* on the ruminal microbial relative abundances (Family level) evaluated *in vitro*

	Control	RS	HA	SEM	Contrast *P*-value	
					Control *vs* RS	Control *vs* HA
Methanobacteriaceae	0.200	0.300	0.200	0.081	0.4502	0.999
Corynebacteriaceae	0.000	0.000	0.900	0.519	0.999	0.3081
Nocardiaceae	0.050	0.000	18.500	10.681	0.997	0.309
Cellulomonadaceae	0.100	0.750	9.100	0.436	0.370	0.0007
Microbacteriaceae	0.000	0.000	0.900	0.519	0.999	0.308
Micrococcaceae	32.900	0.000	2.450	19.047	0.309	0.340
Sanguibacteraceae	0.000	0.000	2.100	1.212	0.999	0.308
Coriobacteriaceae	0.400	0.400	0.350	0.064	0.999	0.622
Prevotellaceae	7.400	9.050	5.350	3.190	0.738	0.680
Rikenellaceae	2.500	2.300	1.600	0.355	0.717	0.171
Bacillaceae	0.050	0.100	0.100	0.028	0.308	0.309
Paenibacillaceae	0.250	0.500	0.450	0.227	0.493	0.577
Carnobacteriaceae	3.200	16.750	4.250	5.965	0.206	0.908
Streptococcaceae	28.500	42.700	33.150	10.770	0.420	0.780
Lachnospiraceae	3.000	2.250	1.850	0.917	0.603	0.440
Christensenellaceae	11.550	14.000	11.700	5.559	0.775	0.986
Ruminococcaceae	2.250	2.400	2.000	0.693	0.888	0.815
Erysipelotrichaceae	1.000	0.650	0.550	0.122	0.136	0.080
Acidaminococcaceae	0.700	0.550	0.650	0.123	0.450	0.791
Veillonellaceae	0.500	1.300	0.700	0.282	0.139	0.651
Succinivibrionaceae	2.650	3.100	1.650	1.060	0.783	0.552
Others	2.800	2.900	1.500	0.624	0.917	0.237

RS= red seaweed; HA= herbal additive; SEM= standard error of the mean. HA= Peptasan® herbal additive.

Discussion

In vitro studies have been used to identify products that can reduce ruminal methane, however, *in vivo* evaluations are required to confirm the results in animal health and production[39]; likewise, factors that can alter the results must be taken into account to design sampling and incubation conditions[25] that reveal useful information.

There is global interest in finding feed additives to reduce ruminal methane emissions, using metabiotic methane inhibitors[40], seaweeds[41], and other phytobiotics. Bunglavan *et al*[42] evaluated various herbs and their extracts regarding their potential to reduce methane production. According to these authors, extracts of *Acacia concinna* had the highest potential for inhibiting methanogenesis compared to those of the other herbs, confirming the results found here.

Palmaria palmata contained high levels of alkanes (Table 1), such as heptadecane, which, similar to 10, 14-trimethyl-2-pentadecanone, have antidiarrheal properties by deactivating the enzyme arginine decarboxylase in *Campylobacter*[43] in addition sea red has other metabolites with antibacterial effects such as 6 1,2-benzenedicarboxylic acid, diisooctyl ester[44], 2(4H)-benzofuranone, 5,6,7,7a-tetrahydro- 4,4,7a-trimethyl-, (R)[45] Silane, 1,4-phenylenebis-trimethyl-[46] and 3,7,11,15-Tetramethyl- 2-hexadecen-1-ol[47]. These findings explain the changes in the VFA levels, even though there were few changes in the detected families (Table 4). Therefore, it is possible that the metabolites of the red algae have affected the ruminal protozoa which are implied in methanogenesis and the fermentation pattern[48]. *Palmaria palmata* also contains a low-molecular-weight halogenated compound, namely 2(3H)-furanone, dihydro-5-tetradecyl, which may be responsible for ruminal methane reduction. The fermentation pattern suggests that the halogenated compounds reduced ruminal methane by inhibition genes expressing enzymes involved in methane synthesis, such as methyl-coenzyme M reductase (MCR)[49], in a similar way as the 3-NOP inhibitor, designed to inhibit MCR, which is essential for archaeal methanogenesis[40]. Methane can be reduced not necessarily associated to an abundance reduction of *Archaea*, but to interference in a step of methanogenesis. The red seaweed compounds modified rumen fermentation and VFA patterns inhibiting the hydrogenotrophic methanogenesis pathway[20]. *In vitro* methane reduction were significant (98.9 %[12] and 99 %[5]) with *Asparagopsis taxiformis*, whereas *in vivo* reduction was lower, among 29.4 and 34.4 %[13].

Methane reduction with the herbal additive could be explained by the antimethanogenic action of saponins[50] and by the presence of other metabolites with antibacterial and antiprotozoal effects, such as alkaloids, flavonoids, tannins, phenolic compounds, and terpenoids[51,52], which may modulate rumen fermentation and improve nutrient use[53]. In the present study, the abundance of *Methanobacteriaceae* was not significantly affected. The herbal additive also contains other secondary metabolites, among which are alkanes such as dotriacontane, triacontane, and docosane[7], with antiviral[54] and antibacterial properties[55]. Overall, the antibacterial activity of this herbal additive could be attributed to metabolites such as triacontane[55], oleic acid[56], docosane[57], cinnamyl cinnamate[58], hexadecanoic acid methyl ester[59], and tymol[60-63]. The saponin content in the herbal additive is 15 % of DM[3]. Additives with high saponin levels have been evaluated in lambs[6] and calves[7] and resulted in reduced animal performance at higher dietary concentrations, reducing DM digestibility linearly with an increasing additive concentration[6]. However, in other studies, saponin-rich additives improved lamb performance in un-dewormed lambs[64] and immune system responses in calves[7], which can be explained by their anthelmintic properties[3]. At low concentrations in the diet, saponins are beneficial for ruminants by improving fermentation efficiency, decreasing protozoan populations, and, consequently, reducing the abundances of methanogenic *Archaea*[8-11]. The antiprotozoal effects of saponins are due to their ability to form irreversible complexes with the lipidic protozoan cell membrane, causing membrane rupture, cell lysis, and death[65].

Comparatively, the magnitude of methane reduction with *Palmaria palmata* was greater than that observed with the herbal additive. Abbott *et al*[41] observed CH_4 mitigation effects with the addition of some red seaweed species (*Gigartina* spp., *Gracilaria vermiculophylla*, *Laurencia filiformis*, and *Hypnea pannosa*) at concentrations between 40 and 60 % (and at high dry matter concentrations). In contrast, the red alga *Asparagopsis taxiformis* reduced the CH_4 by approximately 100 % at concentrations above and around 1 % of dry matter. Machado *et al*[5] also reported that *Asparagopsis taxiformis* at 2 % of dry matter or the halogenated methane analog bromoform (5 μM) *in vitro* reduced methane production by more than 99 %. Further *in vivo* studies are needed to determine the inclusion levels of *Palmaria palmata* that can reduce methane emission without affecting productive performance[20,66]. Since *in vitro* results show a propionate reduction, the *Palmaria palmata* inclusion could reduce the energy from VFA for ruminants, therefore it is necessary to evaluate whether this could be compensated for the energy saved in ruminal methane.

Stoichiometric estimates of methane and CO_2 with the simplified Van Soest equations[67] resulted in values similar to those show in Table 2 for the CO_2 levels of the different treatment, whereas for CH_4, the estimated values were 29 % higher for the control. With the addition of *Palmaria palmata* and the herbal additive, the estimated values were 240 % and 99 % higher, respectively, than the observed ones. This indicates that H is being incorporated in alternative pathways in the presence of both additives, presumably in reductive acetogenesis (reduction of CO_2 with H_2 to acetate), in NH_4 cycling, and in the biohydrogenation of unsaturated fatty acids[68], pathways that are not considered with the stoichiometric model, which could explain the higher concentration of acetate observed with red seaweed. Other marine algae (*Macrocystis pyrifera*, *Ulva* spp., *Mazzaella* spp.; red algae) have shown increases or reductions in VFA concentration, have been consistent in increasing propionate *in vitro*[69].

Both additives contain unsaturated fatty acids, where double bonds follow biohydrogenation, which requires H in the rumen[70]. The herbal additive contains oleic acid[7], whereas *Palmaria palmata* contains pentadecanoic acid, 14-methyl -, methyl ester (Table 1), and may contribute partially to the reduction of ruminal CH_4 production[71]. In future studies it will be important to characterize the contaminating heavy metals in seaweed because some are used in human food[72] and research possibly will be included in ruminants' feed.

In their *in vitro* study, Choi *et al*[73] evaluated four red seaweed species (*Amphiroa anceps*, *Chondracanthus tenellus*, *Grateloupia elliptica*, and *Gracilaria parvispora*) and reported methane reduction levels between 4.6 % and 51.5 %, indicating that different species can vary in their fermentation potential. Terry *et al*[74] reported that the seaweed *A. taxiformis in vitro* reduced the concentration of CH_4 by up to 95.1 %, reducing the total VFA concentration (reducing the proportion of acetate and increasing that of butyrate). In a study[75], *A. taxiformis* decreased the methane concentration and archaeal abundance *in vitro*, also reducing the total VFA level (by reducing acetate and increasing butyrate concentrations). Ahmed *et al*[76] included *A. taxiformis* at 2.5 % *in vitro* and found an 80 % reduction of CH_4 without affecting the total VFA concentration. Other seaweeds have shown inconsistency in VFA concentration response but consistently have increased propionate *in vitro*[69]. In the present study, methane was reduced by 29 % with the herbal additive and by 56 % with the red seaweed compared to the control. The total VFA concentration was increased with *Palmaria palmata* addition, which could be a positive aspect because dietary interventions that inhibit methanogenesis are generally associated with a decreased VFA production[76].

The fact that both products did not reduce digestibility is important because this variable is crucial in ruminant performance[77]. In another study, the herbal additive had no effect on the *in vivo* total DM digestibility in lambs receiving 4 g/d[64]. Few studies have investigated the effects of *Palmaria* on *in vivo* digestibility, and there is no information on ruminant performance. However, in one *in vitro* study, *Palmaria palmata* increased DM digestibility, standing out among other seaweed species[22]. Regarding the effects of *Asparagopsis taxiformis*, it was reported[41] that its inclusion at 10 % had a detrimental effect on ruminal organic matter degradability. Some results[78] indicate that *Macrocystis pyrifera* and *Ulva* spp. have no impact on *in vitro* DM digestibility, whereas *Mazzaella* spp. reduces DM digestibility.

The biological role played of family *Sanguibacteraceae* is not known as well as *Cellulomonadaceae*, although these results showed a significant increase in this last bacterial family in response to the herbal additive, so it would be advisable to continue investigating its metabolic potential as part of the ruminal microbial community. The biological significance of some other bacterial families such as *Micrococcaceae, Microbacteriaceae* and *Corynebacteriaceae*, is not clear, but they have been related with the production of volatile sulphur compounds (VSCs) -except thioesters-[79], nevertheless the data did not allow the detection of statistically significant differences among them in response to treatments. In previous studies, no significant differences were observed regarding the abundances of cellulolytic bacterial families[80,81], which are associated with a higher proportion of acetate[82], which coincides with the non-affected digestibility. The decrease in propionate could be attributed to the reduction in the abundance of species of the family *Rikenellaceae*, capable to produce succinate, which is subsequently catabolized to propionate(82,83). These results confirm the potential of the evaluated products to provide benefits for ruminant performance. The potential to modify ruminal fermentation and reduce methane emissions should improve the feed efficiency in ruminants(52).

Conclusions and implications

The results confirm that the herbal product based on *Acacia concinna* and the red algae *Palmaria palmata* have metabolites that can reduce ruminal methane by different mechanisms of action. The methane reductive potential of both additives must be corroborated with *in vivo* studies to verify if the fermentation profile could be a drawback for the energy obtained from volatile fatty acids.

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Literature cited:

1. Benaouda M, González-Ronquillo M, Molina LT, Castelán-Ortega OA. Estado de la investigación sobre emisiones de metano entérico y estrategias de mitigación en América Latina. Rev Mex Cienc Agric 2017;8(4):965-74.

2. Ku-Vera JC, Jiménez-Ocampo R, Valencia-Salazar SS, Montoya-Flores MD, Molina-Botero IC, Arango J, *et al*. Role of secondary plant metabolites on enteric methane mitigation in ruminants. Front Vet Sci 2020;7.

3. Sánchez-Hernández C, Castañeda-Gómez CJ, Trejo-Castro L, Mendoza-Martínez G, Gloria-Trujillo A. Evaluation of a feed plant additive for coocidiosis control in broilers herbals for coccidiosis control. Braz J Poult Sci 2019;21(01):eRBCA-2019-0846.

4. Becker PM, van Wikselaar PG, Franssen MCR, de Vos RCH, Hall RD, Beekwilder J. Evidence for a hydrogen-sink mechanism of (+) catechin-mediated emission reduction of the ruminant greenhouse gas methane. J Metabolomics 2014;10(2):179-89.

5. Machado L, Tomkins N, Magnusson M, Midgley DJ, de Nys R, Rosewarne CP. *In vitro* response of rumen microbiota to the antimethanogenic red macroalga *Asparagopsis taxiformis*. Microb Ecol 2018;75(3):811-818.

6. Orzuna-Orzuna JF, Dorantes-Iturbide G, Lara-Bueno A, Mendoza-Martínez GD, Miranda-Romero LA, Hernández-García PA. Growth performance, carcass characteristics, and blood metabolites of lambs supplemented with a polyherbal mixture. Animals 2021;11(4):955.

7. Lee-Rangel HA, Mendoza-Martinez GD, Martínez-García JA, Espinosa-Ayala E, Hernández-García PA, Cifuentes-López RO, *et al*. An Indian polyherbal phytogenic source improved blood serum biochemistry and immune response of dairy calves. Food Agric Immuno 2022;33(1):97-112.

8. Patra AK, Saxena J. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 2010;71(11):1198-222.

9. Zhou YY, Mao HL, Jiang F, Wang JK, Liu JX, McSweeney CS. Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. Anim Feed Sci Technol 2011;166-167:93-100.

10. Goel G, Makkar HPS. Methane mitigation from ruminants using tannins and saponins. Trop Anim Health 2012;44(4):729-739.

11. Ortiz D, Posada S, Noguera R. Efecto de metabolitos secundarios de las plantas sobre la emisión entérica de metano en rumiantes. Livest Res Rural Dev 2014;26(11).

12. Machado L, Magnusson M, Paul NA, de Nys R, Tomkins N. Effects of marine and freshwater macroalgae on *in vitro* total gas and methane production. PLOS One 2014;9(1):e85289.

13. Roque BM, Salwen JK, Kinley R, Kebreab E. Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. J Clean Prod 2019;234:132-138.

14. Stefenoni HA, Räisänen SE, Cueva SF, Wasson DE, Lage CFA, Melgar A, *et al*. Effects of the macroalga *Asparagopsis taxiformis* and oregano leaves on methane emission, rumen fermentation, and lactational performance of dairy cows. J Dairy Sci 2021;104(4):4157-4173.

15. Kinley RD, Tan S, Turnbull J, Askew S, Harris J, Roque BM. Exploration of methane mitigation efficacy using *Asparagopsis*-derived bioactives stabilized in edible oil compared to freeze-dried *Asparagopsis in vitro*. Am J Plant Sci 2022;13(7):1023-41.

16. Ponte JMS, Seca AML, Barreto MC. *Asparagopsis* genus: What we really know about Its biological activities and chemical composition. Molecules 2022;27(6):1787.

17. Li X, Norman HC, Kinley RD, Laurence M, Wilmot M, Bender H, *et al*. *Asparagopsis taxiformis* decreases enteric methane production from sheep. Anim Prod Sci 2018;58(4):681-688.

18. Kinley RD, Martinez-Fernandez G, Matthews MK, de Nys R, Magnusson M, Tomkins NW. Mitigating the carbon footprint and improving productivity of ruminant livestock agriculture using a red seaweed. J Clean Prod 2020;259:120836.

19. Roque BM, Venegas M, Kinley RD, de Nys R, Duarte TL, Yang X, *et al*. Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. PLOS One 2021;16(3):e0247820.

20. Min BR, Parker D, Brauer D, Waldrip H, Lockard C, Hales K, *et al.* The role of seaweed as a potential dietary supplementation for enteric methane mitigation in ruminants: Challenges and opportunities. Anim Nutr 2021;7(4):1371-1387.

21. Morais T, Inácio A, Coutinho T, Ministro M, Cotas J, Pereira L, *et al*. Seaweed potential in the animal feed: A review. J Mar Sci Eng 2020;8(8):559.

22. Molina-Alcaide E, Carro MD, Roleda MY, Weisbjerg MR, Lind V, Novoa-Garrido M. *In vitro* ruminal fermentation and methane production of different seaweed species. Anim Feed Sci Technol 2017;228:1-12.

23. Werner A, Dring M. Cultivating *Palmaria palmata*: Irish Sea Fisheries Board Dublin; 2011.

24. Grote B. Recent developments in aquaculture of *Palmaria palmata* (Linnaeus) (Weber & Mohr 1805): cultivation and uses. Rev Aqua 2019;11(1):25-41.

25. Yáñez-Ruiz DR, Bannink A, Dijkstra J, Kebreab E, Morgavi DP, O'Kiely P, *et al*. Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants—a review. Anim Feed Sci Technol 2016;216:1-18.

26. Roque-Jiménez JA, Mendoza-Martínez GD, Vázquez-Valladolid A, Guerrero-González ML, Flores-Ramírez R, Pinos-Rodriguez JM, *et al*. Supplemental herbal choline increases 5-hmC DNA on whole blood from pregnant ewes and offspring. Animals 2020;10(8):1277.

27. Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, France J. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Technol 1994;48(3):185-197.

28. Menke HH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim Res Dev 1988;28:7-55.

29. Bartha R, Pramer D. Features of a flask and method for measuring the persistence and biological effects of pesticides in soil. Soil Sci 1965;100(1):68-70.

30. Hegarty R, Leng R, Nolan JV. Measurement of methane production rate in the rumen using isotopic tracers. Springer; 2007.

31. Ramírez-Díaz R, Pinto-Ruiz R, Miranda-Romero LA, La O, Arias MA, Hernández-Sánchez D, *et al*. Predicción de metano de dos frutos arbóreos por cromatografía de gases y gas i*n vitro*. Ecosis Recur Agropecu 2023;10(3).

32. Erwin E, Marco GJ, Emery E. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. J Dairy Sci 1961;144:1768–1771.

33. Mendoza-Martínez GD, Hernández-García PA, Díaz-Galván C, Razo-Ortiz PB, Ojeda-Carrasco JJ, Sánchez-López N, *et al*. Evaluation of Increasing dietary concentrations of a multi-enzyme complex in feedlot lambs' rations. Animals 2024;14(8):1215.

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

35. 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):33533-6.

36. Herrera-Haro JG, García-Artiga C, Santoyo-Brito E. Bioestadística en ciencias veterinarias: Procedimientos de análisis de datos con SAS. Bioestadística en ciencias veterinarias: procedimientos de análisis de datos con SAS. 2010.

37. Pinos-Rodríguez JM, González-Muñoz SS, Mendoza-Martínez GD, Martínez-Garza Á. Análisis estadístico de experimentos de digestibilidad *in vitro* con forrajes. Interciencia 2002;27(3):143-146.

38. Mendoza-Martínez GD, Hernández-García PA, Plata-Pérez FX, Martínez-García JA, Arcos-García JL, Lee-Rangel HA. Nutrición animal cuantitativa. Editorial CBS. Universidad Autónoma Metropolitana. México. 2022.

39. Dijkstra J, Kebreab E, Bannink A, France J, López S. Application of the gas production technique to feed evaluation systems for ruminants. Anim Feed Sci Technol 2005;123-124:561-578.

40. Pitta DW, Indugu N, Melgar A, Hristov A, Challa K, Vecchiarelli B, *et al*. The effect of 3-nitrooxypropanol, a potent methane inhibitor, on ruminal microbial gene expression profiles in dairy cows. Microbiome 2022;10(1):146.

41. Abbott DW, Aasen IM, Beauchemin KA, Grondahl F, Gruninger R, Hayes M, *et al*. Seaweed and seaweed bioactives for mitigation of enteric methane: Challenges and opportunities. Animals 2020;10(12):2432.

42. Bunglavan S, Valli C, Ramachandran M, Balakrishnan V. Effect of supplementation of herbal extracts on methanogenesis in ruminants. Livest Res Rural Dev 2010;22(11):216.

43. Nayak S, Sahu S, Biswal MK, Dash S, Parida S, Pattanayak S. *Eclipta alba* L. derived phytochemicals against *Campylobacter* causing diarrhea. J Pharm Res Int 2020;32:108-111.

44. Fadipe L, Haruna A, Mohammed I. Antibacterial activity of 1, 2-benzenedicarboxylic acid, dioctyl ester isolated from the ethyl acetate soluble sub-portion of the unripe fruits of *Nauclea latifolia*. Int J Pure Appl Biosci 2014;2(1):223-230.

45. Akhbari M, Batooli H, Kashi FJ. Composition of essential oil and biological activity of extracts of *Viola odorata* L. from central Iran. Iran. Nat Prod Res 2012;26(9):802-809.

46. Alexpandi R, Ponraj JG, Swasthikka RP, Abirami G, Ragupathi T, Jayakumar R, *et al*. Anti-QS mediated anti-infection efficacy of probiotic culture-supernatant against *Vibrio campbellii* infection and the identification of active compounds through *in vitro* and *in silico* analyses. Biocatal Agric Biotechnol 2021;35:102108.

47. Yu J, Lei J, Yu H, Cai X, Zou G. Chemical composition and antimicrobial activity of the essential oil of *Scutellaria barbata*. Phytochemistry 2004;65(7):881-884.

48. McAllister TA, Newbold CJ. Redirecting rumen fermentation to reduce methanogenesis. Aust J Exp Agric 2008;48(2):7-13.

49. Oremland RS, Capone DG. Use of “Specific” Inhibitors in biogeochemistry and microbial ecology. In: Marshall KC, editor. Adv Microb Ecol. Boston, MA: Springer US; 1988.

50. Ugbogu EA, Elghandour MMMY, Ikpeazu VO, Buendía GR, Molina OM, Arunsi UO, *et al.* The potential impacts of dietary plant natural products on the sustainable mitigation of methane emission from livestock farming. J Clean Prod 2019;213:915-925.

51. Kumar M, Kumar V, Roy D, Kushwaha R, Vaiswani S. Application of herbal feed additives in animal nutrition-a review. Int J Livest Res 2014;4(9):1-8.

52. Kuralkar P, Kuralkar SV. Role of herbal products in animal production – An updated review. J Ethnopharmacology 2021;278:114246.

53. Tajodini M, Moghbeli P, Saeedi H, Effati M. The effect of medicinal plants as a feedadditive in ruminant nutrition. Iran J Appl Anim Sci 2014;4(4).

54. Chathuranga K, Weerawardhana A, Dodantenna N, Ranathunga L, Cho WK, Ma JY, *et al.* Inhibitory effect of *Sargassum fusiforme* and its components on replication of respiratory syncytial virus *in vitro* and *in vivo*. Viruses 2021;13(4):548.

55. Amudha P, Jayalakshmi M, Pushpabharathi N, Vanitha V. Identification of bioactive components in *Enhalus acoroides* seagrass extract by gas chromatography-mass spectrometry. Asian J Pharm Clin Res 2018;11(10):313-315.

56. Farhan SR, AL-Azawi AH, Salih WY, Abdulhassan AA. The antibacterial and antioxidant activity of *Moringa oleifera* seed oil extract against some foodborne pathogens. Indian J Forensic Med Toxicol 2021;15(4):2529-2538.

57. Uma B, Parvathavarthini R. Antibacterial effect of hexane extract of sea urchin, *Temnopleurus alexandri* (Bell, 1884). Int J Pharmtech Res 2010;2(3):1677-1680.

58. Sahinler N, Kaftanoglu O. Natural product propolis: chemical composition. Nat Prod Res 2005;19(2):183-188.

59. Shaaban MT, Ghaly MF, Fahmi SM. Antibacterial activities of hexadecanoic acid methyl ester and green-synthesized silver nanoparticles against multidrug-resistant bacteria. J Basic Microbiol 2021;61(6):557-568.

60. Acevedo AM, Castañeda ML, Blanco KM, Cardenas CY, Reyes JA, Kouznetsov VV, *et al.* Composición y capacidad antioxidante de especies aromáticas y medicinales con alto contenido de timol y carvacrol. Sci Technol 2007;13(33):125-128.

61. Gallegos-Flores PI, Bañuelos-Valenzuela R, Delgadillo-Ruiz L, Meza-López C, Echavarría-Cháirez F. Actividad antibacteriana de cinco compuestos terpenoides: carvacrol, limoneno, linalool, α-terpineno y timol. Trop Subtrop Agroecosyst 2019;22(2):241-248.

62. Du E, Gan L, Li Z, Wang W, Liu D, Guo Y. *In vitro* antibacterial activity of thymol and carvacrol and their effects on broiler chickens challenged with *Clostridium perfringens*. J Anim Sci Biotechnol 2015;6(1):58.

63. Di Pasqua R, Mamone G, Ferranti P, Ercolini D, Mauriello G. Changes in the proteome of *Salmonella enterica* serovar Thompson as stress adaptation to sublethal concentrations of thymol. Proteomics 2010;10(5):1040-1049.

64. Mejia-Delgadillo M, Lee-Rangel H, Hernandez-Garcia P, Vazquez-Valladolid A, Mendez-Cortes H, Guerra-Liera J, *et al.* Effect of a polyherbal additive on performance and parasite infection of hair creole ewes. Indian J Anim Res 2021;1(1-5).

65. Francis G, Kerem Z, Makkar HPS, Becker K. The biological action of saponins in animal systems: a review. Br J Nutr 2002;88(6):587-605.

66. Jard G, Marfaing H, Carrère H, Delgenes JP, Steyer JP, Dumas C. French Brittany macroalgae screening: Composition and methane potential for potential alternative sources of energy and products. Bioresour Technol 2013;144:492-498.

67. Van Soest P. Nutritional ecology of the ruminant: Cornell University Press; 1994.

68. Ungerfeld EM. Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. Front Microbiol 2020;11. https://doi.org/10.3389/fmicb.2020.00589

69. De la Cruz Gómez AG, Campos-García H, Mendoza GD, García-López JC, Álvarez-Fuentes G, Hernández-García PA, *et al.* Macroalgae compound characterizations and their effect on the ruminal microbiome in supplemented lambs. Vet Sci 2024;11(12):653.

70. Jenkins TC, Wallace RJ, Moate PJ, Mosley EE. Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J Animal Sci 2008;86(2):397-412.

71. Ungerfeld EM. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. Front Microbiol 2015;6.

72. Lindenmayer R, Lu L, Eivazi F, Afrasiabi Z. Atomic spectroscopy-based analysis of heavy metals in seaweed species. Appl Sci 2023;13(8):4764.

73. Choi Y, Lee S, Kim H, Eom J, Jo S, Guan L, *et al.* Red seaweed extracts reduce methane production by altering rumen fermentation and microbial composition *in vitro*. Front Vet Sci 2022;9. https://doi.org/10.3389/fvets.2022.985824

74. Terry SA, Krüger AM, Lima PMT, Gruninger RJ, Abbott DW, Beauchemin KA. Evaluation of rumen fermentation and microbial adaptation to three red seaweeds using the rumen simulation technique. Animals 2023;13(10):1643.

75. O'Hara E, Terry SA, Moote P, Beauchemin KA, McAllister TA, Abbott DW, *et al*. Comparative analysis of macroalgae supplementation on the rumen microbial community: *Asparagopsis taxiformis* inhibits major ruminal methanogenic, fibrolytic, and volatile fatty acid-producing microbes *in vitro*. Front Microbiol 2023;14.

76. Ahmed E, Suzuki K, Nishida T. Micro- and Macro-Algae combination as a novel alternative ruminant feed with methane-mitigation potential. Animals 2023;13(5):796.

77. Mendoza-Martínez G, Plata-Pérez F, Espinosa-Cervantes R, Lara-Bueno A. Manejo nutricional para mejorar la eficiencia de utilización de la energía en bovinos. Universidad y Ciencia 2008;24(1):75-87.

78. Lee-Rangel H, Roque-Jiménez J, Cifuentes-López R, Álvarez-Fuentes G, Cruz-Gómez A, Martínez-García J, *et al.* Evaluation of three marine algae on degradability, *in vitro* gas production, and CH4 and CO_2 emissions by ruminants. Fermentation 2022;8(10):511.

79. Montel MC, Buchin S, Mallet A, Delbes-Paus C, Vuitton DA, Desmasures N, *et al*. Traditional cheeses: Rich and diverse microbiota with associated benefits. Int J Food Microbiol 2014;177:136-154.

80. Geier RR, Kwon IH, Cann IK, Mackie RI. Interspecies hydrogen transfer and its effects on global transcript abundance in *Ruminococcus albus*, a predominant fiber-degrading species in the rumen. FASEB J 2016;30(S1):1102.1-.1. https://doi.org/10.1096/fasebj.30.1_supplement.1102.1.

81. Ramírez-Bribiesca J. Eventos químicos-fisiológicos del metano en los rumiantes. Agro Productividad 2018;11(2).

82. Andrade BGN, Bressani FA, Cuadrat RRC, Cardoso TF, Malheiros JM, de Oliveira PSN, *et al.* Stool and ruminal microbiome components associated with methane emission and feed efficiency in Nelore beef cattle. Front Genet 2022;13.

83. Bach A, López-García A, González-Recio O, Elcoso G, Fàbregas F, Chaucheyras-Durand F, *et al.* Changes in the rumen and colon microbiota and effects of live yeast dietary supplementation during the transition from the dry period to lactation of dairy cows. J Dairy Sci 2019;102(7):6180-6198.