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Can Marandu Grass (*Urochloa brizantha*) Extract Modulate Methanogenesis and Rumen Microbiota?

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Abstract: *Urochloa* spp. are the most important grasses for ruminants in Brazil and contain secondary metabolites, mainly saponins. *Urochloa brizantha* extracts (ethanolic EE and hydroalcoholic HE with 3.62 and 5.38 mg protodioscin mL^{-1}, respectively) were developed to verify their potential as additives for ruminant nutrition. The in vitro gas production technique was used to evaluate ten treatments in a completely randomized factorial arrangement ($2 \times 4 + 2$), where the main effects were two extracts (EE and HE); four levels (50, 100, 150, and 200 mL of the extract kg^{-1} of DM), plus two controls (one positive with 25 ppm of monensin and another with no additives). The extracts EXT (EE and HE) produced a higher proportion of acetate (C2) and lower propionate (C3) than CTL, reflected in a 31% higher C2:C3 ratio. However, there was no significant difference ($p > 0.05$) between the treatments for methane production parameters. *Archaea* and *Ruminococcus'* relative gene expressions were higher in EE than in HE; however, the protozoa opposite occurred, HE was higher than EE. *Fibrobacter succinogenes* were 33% lower in EXT than in CTL. The addition of these extracts in a sheep diet increased the production of SCFA and decreased *Fibrobacter succinogenes* without altering the methane and archaeal population.

Keywords: greenhouse gas; ruminant nutrition; saponin; secondary metabolites

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1. Introduction

Enteric methane (CH_4) emissions constitute 6% of global anthropogenic greenhouse gas (GHG) emissions [1], and also contribute between 2 and 12% of energy loss by animals [2]. According to D'Aurea et al. [3], the projection of the global population reaching twelve billion by 2050 implies an increase in food production, thereby escalating concerns regarding safety and sustainability [4]. In this context, the utilization of feed additives to improve diet utilization efficiency and animal performance is very important.

Among the techniques used, monensin is the most commonly used feed additive in feedlot diets in Brazil [5]. However, due to concerns about residues and the development of antibiotic resistance in humans (cross-resistance) [6], their use has been banned in the European Union banned since 2006 [7]. Consequently, there has growing interest in natural alternatives, such as plant extracts that contain bioactive compounds [8].

According to Zhang et al. [6], bioactive compounds have a great potential in mitigation, offering advantages such as better efficiency and a low risk of host toxicity or residues, in contrast to ionophores. Saponins have emerged as important bioactive compounds, prevalent in the plant kingdom, and are categorized into triterpenoid and steroidal groups [9].

These groups exhibit medicinal attributes, including anti-inflammatory, antimicrobial, antifungal, insecticidal, and anticancer properties [8].

El Hazzam et al. [10] considered saponin as an anti-nutritional factor. However, saponins can modulate rumen fermentation, especially by reducing protein degradation, urea, and ammoniacal nitrogen (NH_3-N) concentrations in the rumen, thereby promoting an influx of amino acids into the small intestine [11] and exhibiting antiprotozoal activity [12]. Thus, studies using saponins as additives in ruminant feed have observed their potential to increase feed digestibility and reduce methane production [13].

Several studies have evaluated the use of steroidal saponins as additives in ruminant feed, and the findings have been mixed [13–16]. Pen et al. [15] found that *Yucca Schidigera* (YS) had a dose-dependent effect on methane mitigation, with a reduction of 42%. Other authors showed that YS extract did not affect methane production, but resulted in a 3.06% increase in the proportion of propionic acid (C3) [16]. Additionally, the inclusion of fenugreek (*Trigonella foenum-graecum* L.) in a heifer's diet led to a 33.23% reduction in methane production, an increased propionate proportion, and a decrease in the acetate: propionate ratio, resulting in a greater amount of microbial protein [17].

Forage plants, especially of the *Urochloa* genus, are the main source of feed for ruminants in Brazil, accounting for approximately 85% of the pastures cultivated in Brazil [18]. This forage is known to contain steroidal saponins [19] and according to Pires et al. [20], four steroidal saponins like diosgenin, yamogenin, dioscin, and 3-O-{a-L-rhamnopyranosyl-(1→4)-[a-L-rhamnopyranosyl-(1→2)]-b-D-glucopyranosyl}-25(S)-spirost-5-en-3b-ol were found in the leaves of *Urochloa decubens*. Brum et al. [19] also found protodioscin in *U. decumbens* and *U. brizantha*. Meanwhile, Oliveira et al. [21] and Feitoza et al. [22], when studying *U. humidicola* roots, identified dioscin, pennongenin-3-O-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, and floribundasaponin.

Within the *Urochloa* genus, the species *U. brizantha* cv. Marandu, known as Marandu grass or "braquiarão", is more widespread in Brazilian livestock farming due to its high productivity and adaptability [18]. Using Marandu grass extracts allows for a precise assessment of bioactive compounds, such as protodioscin, and their effects on rumen fermentation. Additionally, these extracts can improve feed efficiency and reduce GHG production. The *Urochloa humidicola* extract was used in an in vitro test, finding that concentrations of 75, 150, and 250 g L^{-1} reduced CH_4 production at the expense of dry matter degradation [23]. However, to our knowledge, no study has evaluated the production of *Urochloa brizantha* extract and its effect on in vitro ruminal fermentability.

Therefore, the specific mechanisms by which the steroidal saponins in *Urochloa brizantha* extracts influence rumen microbial populations and methane production are not fully understood and warrant further investigation. Thus, in this study, concentrations of extracts of *Urochloa brizantha* containing steroidal saponins were evaluated with regard to methane production, short-chain fatty acids (SFCAs), ammonia nitrogen, and rumen microbiota using an in vitro gas production technique.

2. Materials and Methods

The study was approved by the ethics committee of the College of Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil (Protocol number CEUA: 1344101121).

2.1. Inoculum Donors and Substrate

Eight rumen-cannulated adult Santa Inês × Dorper sheep (41.11 ± 8.08 kg BW) were previously adapted (14 days) to the experimental diet with 70% corn silage, 30% concentrate (composed of 63% of ground corn grain, 31% soybean meal, 2% calcitic limestone, and 4% mineral supplement, on a dry matter basis), and free access to a mineral supplement and fresh water.

The animal diet was dry and ground in a Willey mill with a 1 mm sieve as a substrate for methanogenesis bioassay. The substrate was analyzed and presented the following

composition: dry matter, 36.1 g kg^{-1} (DM; ID 930.15); mineral matter, 55.6 g kg^{-1} DM (ash; ID 942.05); crude protein, 114.9 g kg^{-1} DM (CP; ID 954.01); ether extract, 29.1 g kg^{-1} DM (EE; ID 920.39); neutral detergent fiber, 438.6 g kg^{-1} DM (NDF; ID 973.18), and acid-detergent fiber, 245.1 g kg^{-1} DM (ADF; ID 973.18) according to the AOAC [24].

On the day of incubation, rumen content was collected before morning feeding and four inocula were prepared, each from two donors, then the solid and liquid phases were homogenized in a 1:1 ratio in a blender for 10 s and filtered through two layers of cotton cloth, according to the methodology proposed by Bueno et al. [25].

2.2. Extracts and Treatments

The extraction of *U. brizantha* cv. Marandu was prepared using pressurized liquid with ethanol (99.5% EE) and hydroalcohol (70% HE). The saponin concentrations in the extracts (EE and HE) were determined and saponin was quantified with high-performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry as described by Lee et al. [26] when using protodioscin (Sigma Aldrich Inc., G0299, St. Louis, MO, USA) as a stander. The calibration curve used nine levels, with concentrations of 0.10 to 12 µg/mL.

Ten treatments were designed to evaluate the two extracts (ethanolic EE and hydroalcoholic HE); with four levels of inclusion (dosages) 50, 100, 150, and 200 mL kg^{-1} DM, and two controls, one positive (MON, 25 ppm of monensin) and another negative (CTL, with no additives) in a completely randomized factorial arrangement (2 × 4 + 2). The dosages used corresponded to 0.37, 0.75, 1.12, and 1.50 mg saponin (protodioscin) g^{-1} DM.

2.3. Methanogenesis Assay

To evaluate the effect of extracts on methanogenesis, a total of 132 glass bottles with a capacity of 160 mL were prepared, with four inocula and eleven treatments (ten tested and a blank) in triplicates.

Each glass bottle was prepared with 0.5 g of substrate placed in filter bags (TNT, 100 g m^{-2}); there was 25 mL of rumen inoculum and 50 mL of nutrient solution in each fermentation flask, according to the semi-automatic in vitro gas production technique proposed by Theodorou et al. [27] and modified by Mauricio et al. [28]. The nutrient solution was composed of micromineral solutions ($CaCl_2.2H_2O$, $MnCl_2.2H_2O$, $CoCl_2.6H_2O$, and $FeCl_3.6H_2O$), macrominerals (Na_2HPO_4, KH_2PO_4, and $MgSO_4.7H_2O$), a buffer (NH_4CO_3 and $NaHCO_3$), reducing agent (cysteine-HCl, 1M NaOH, and $Na_2S.9H_2O$) and an indicator (resazurin), prepared according to the method described by Menke [29].

Pressure in the headspace was measured after 4, 8, 12, 16, 20, and 24 h of incubation using a transducer (Pressure Press Data 800; Piracicaba, SP, Brazil) and 1.0 mL gas samples in duplicate were collected and stored in 10 mL vacutainer tubes (Becton, Dickinson, Franklin Lakes, NJ, USA), composing the sample, then stored in refrigerator until determination of CH_4 characterization by the gas chromatography [30], which was performed immediately after the collection.

The total gas volume produced in each bottle was estimated according to the equation V = (6.4278 × P), where V = volume of gases (mL) and P = measured pressure (psi). This equation is accepted for in vitro experimental conditions for specific methanogenesis bioassays at the Ruminal Fermentability Laboratory.

Following the 24 h incubation, the bottles were placed in containers with ice to stop the fermentation process and 2 mL samples of the ruminal liquid contained in each bottle were collected for the determination of short-chain fatty acids (SCFAs), ammonia nitrogen (NH_3-N), and real-time polymerase chain reaction (qPCR).

The in vitro dry matter degradability (IVDMD) was obtained by measuring the difference between the dry weights before and after incubation (drying was at 105 °C, overnight). The in vitro organic matter degradability (IVMOD) was estimated by the difference in residues (ash) after muffle burning (550 °C) for 4 h. Total gas production (GP) was calculated as the volume of gas (mL) produced after 24 h of incubation, correcting the values of gas production for the corresponding blank, and divided by the amount of DM incubated

(g). The partition factor (PF) was determined by the relationship between the IVDMD (mg) and total gas production (mL) in 24 h, according to Blümmel, Makkar, and Becker [31].

2.4. Methane (CH_4) Determination

The CH_4 quantification was analyzed by gas chromatography (GC-2014, Shimadzu, Kyoto, Japan) equipped with a micropacked column (ShinCarbon® Restek Corporation, Bellefonte, PA, USA), according to the method by Santos et al. [30]. A calibration curve (0, 30, 60, and 90 mL L^{-1}) was produced using methane (99% purity) as the standard. The operational conditions included column and injector temperatures at 100 °C, and flame ionization detectors at 120 °C.

2.5. Short Chain Fatty Acid (SCFA) and Ammonia Nitrogen (NH_3-N) Determination

A short-chain fatty acid (SCFA) profile in the ruminal fluid was performed by gas chromatography (CG–2014; Shimadzu) with injector and detector temperatures of 250 °C; helium was the carrier gas at 8.01 mL/min, and the hydrogen flow to the flame jet was 60 kPa and the synthetic air was 40 kPa [32]. The samples of ruminal fluid were centrifuged at 14,500× *g* for 10 min. The supernatant (800 µL) was transferred to a dry and clean flask with 200 µL formic acid (98–100%) and 100 µL of the internal standard (100 mM 2-ethyl butyric acid, Chem Service Inc., West Chester, PA, USA). Acetic, propionic, isobutyric, butyric, isovaleric, and valeric acid (99.5% purity, Chem Service Inc., West Chester, PA, USA) were used as a quantitative external standard.

Ammonia nitrogen (NH_3-N) concentrations in the rumen fluid were determined by colorimetry using a commercial enzymatic urea kit UREIA UV K056 (Bioclin®, Belo Horizonte, Brazil), and read at 670 nm, according to the methodology developed by Kulasek [33] and adapted by Foldager [34]. In each sample, 10% sodium tungstate was added and then centrifuged at 200× *g* for 15 min. The supernatant was then transferred to another tube with 1 mL of the salicylic acid buffer and 1 mL of sodium hypochlorite oxidizing solution. At the end, the tubes were placed in a water bath at 37 °C until they became green.

2.6. Microbial Quantifications

DNA was isolated from the samples collected from ruminal contents incubated in vitro using the PureLinkTM Microbiota kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's recommendations. The quality and quantity of DNA were measured using a NanoDrop One/One spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and agarose gel electrophoresis.

Quantitative polymerase chain reaction (qPCR) was performed in duplicate using a CFX96TM thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) to estimate the number of methanogenic Archaea, Protozoa, and the total number of bacteria species *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* in the samples (Table 1). A negative control was evaluated on each plate to detect possible contaminants or the formation of primer dimers. The cycling conditions for Archaea and total bacteria were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s, and 79 °C for 10 s; for the protozoa, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens*, the conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Immediately after amplification, a melting curve was performed.

After the qPCR reaction, the bacterial DNA fragments were analyzed using conventional 1% agarose gel electrophoresis. The efficiency of each primer was evaluated based on a linear regression model using serial dilution of a pool of DNA samples. The PCR amplification efficiency was calculated according to the equation E = 10(−1/slope), and relative quantification (RQ) values were determined using the method of Pfaffl [35]. The total 16S rRNA gene amplified by the total bacteria primer was used as the endogenous gene for data normalization [36].

Table 1. Primer set used for quantitative polymerase chain reaction.

Target Species	(F) and (R)	pb	References
Total bacteria	F-CGGCAACGAGCGCAACCC R-CCATTGTAGCACGTGTGTAGCC	130	[36]
Fibrobacter succinogenes	F-GTTCGGAATTACTGGGCGTAAA R-CGCCTGCCCCTGAACTATC	121	[36]
Ruminococcus flavefaciens	F-CGAACGGAGATAATTTGAGTTTACTTAGG R-CGGTCTCTGTATGTTATGAGGTATTACC	132	[36]
Archaea	F-AATTGGAKTCAACGCCGGR R-TGGGTCTCGCTCGTTG	142	[37]
Protozoa	F-GCTTTCGWTGGTAGTGTATT R-CTTGCCCTCYAATCGTWCT	223	[38]

F, forward; R, reverse; [36], Denman e McSweeney. (2006); [37], Poulsen et al. (2013); [38], Sylvester et al. (2004).

2.7. Experimental Design and Statistical Analysis

The experimental design was completely randomized in a factorial arrangement; 2 × 4 + 2, with two types of *U. brizantha* extracts (ethanolic 99% and hydroalcoholic 70%), four concentrations (50; 100; 150; and 200 mL kg^{-1} of DM) plus additional treatments of a positive control (monensin; 25 mg of Rumensin® kg^{-1} DM, Elanco Animal Health, Indianapolis, IN, USA), and a negative control (without additive inclusion), with three replicates in each experimental unit.

The normality of the residuals was assessed using the Shapiro–Wilk test (PROC UNIVARIATE). Statistical analysis was carried out with the GLIMMIX procedure (SAS 9.4; SAS Institute, Cary, NC, USA) using orthogonal contrasts of the effect of grouped treatments:

(1) Effect of monensin (MON) treatment against control (CTL);
(2) Effect of extracts (EXT, the grouping of EE and HE extracts in all doses) concerning the positive control (MON);
(3) Effect of extracts (EXT, the grouping of EE and HE extracts in all doses) concerning the negative control (CTL);
(4) Effect of the type of extract (ethanolic EE and hydroalcoholic HE) at different doses, 50, 100, 150, and 200 mL/kg DM);
(5) Level effect (LVL), comparing each level (50, 100, 150, and 200 mL kg^{-1} DM) against the other levels of extracts;
(6) EXT vs. LVL interaction effect.

Significance was set at $p \leq 0.05$ and the p values obtained for the contrasts are reported.

3. Results

3.1. Methane Productions and Ruminal Degradability

There were no significant differences between the treatments in terms of methane (CH_4) production, in vitro dry matter degradability (IVDMD, %), in vitro organic matter degradability (IVOMD, %), gas production accumulated after 24 h (GP24), and the partitioning factor (PF, mg DMD mL^{-1}) (Table 2).

3.2. Short-Chain Fatty Acids and Ammonia Nitrogen

The in vitro inclusion of extracts in the diet altered the simulated rumen fermentation pattern compared to the control treatment (CTL) (Table 3). The inclusion of extracts (EXTs) showed a higher proportion of acetate (C2) (70.64%) compared to the CTL treatment (63.05%). Conversely, the EXT treatment produced a lower percentage of propionate (C3) (14.10%) compared to the CTL (18.04%), resulting in a significantly higher C2:C3 ratio in the EXT (4.92) compared to the CTL (3.76).

Table 2. Effect of ethanolic (EE) and hydroalcoholic (HE) extracts on methane, degradability, and partitioning factor.

Variable	EE				HE				MON	CTL	SEM	Contrast *p*-Value					
	50	100	150	200	50	100	150	200				1	2	3	4	5	6
CH_4 (mL)	7.13	7.85	6.92	7.16	7.74	7.01	8.38	7.33	6.79	6.39	1.42	0.71	0.45	0.16	0.48	0.56	0.36
CH_4 (%)	7.84	8.68	7.82	7.95	8.60	7.74	8.74	7.66	7.65	7.32	1.00	0.74	0.54	0.23	0.80	0.45	0.34
Net CH_4 (mL)	5.26	5.98	5.05	5.29	5.87	5.14	6.51	5.47	4.93	4.52	1.17	0.70	0.46	0.16	0.48	0.57	0.36
CH_4 (mL g^{-1} DMD)	18.62	21.64	18.52	19.50	20.37	18.28	23.13	19.35	17.20	16.03	4.11	0.75	0.35	0.13	0.67	0.55	0.32
IVDMD (%)	57.19	55.97	55.08	54.81	58.18	55.82	55.92	56.33	55.76	56.69	1.27	0.61	0.77	0.70	0.38	0.96	0.79
IVOMD (%)	56.33	54.40	54.62	54.87	57.19	54.24	54.75	55.05	54.43	56.30	1.52	0.38	0.63	0.48	0.81	0.86	0.99
GP24 (mL g^{-1} DM)	120.5	117.6	112.9	116.1	117.7	116.4	124.0	126.5	109.8	112.1	9.28	0.83	0.27	0.41	0.43	0.71	0.97
PF (mg DMD mL^{-1})	4.93	4.84	4.92	4.72	4.95	4.88	4.64	4.56	5.15	5.07	0.30	0.81	0.20	0.33	0.59	0.58	0.82

EE, ethanolic extract; HE, hydroalcoholic extract; EXT, grouping of EE and HE extracts in all doses; MON, 25 ppm monensin; CTL, control (no additive added); LVL, level effect; SEM, standard error of the mean. DMD, dry matter degradability; IVDMD, in vitro dry matter degradability; IVOMD, in vitro organic matter degradability; GP24, gas production after 24 h; PF, partitioning factor. Contrasts: 1, MON vs. CTL; 2, EXT vs. MON; 3, EXT vs. CTL; 4, EE vs. HE; 5, level effect; 6, effect of EXT vs. LVL interaction.

Table 3. Effect of ethanolic (EE) and hydroalcoholic (HE) extracts on the production of short-chain fatty acids (SCFAs, expressed as molar%) and ammoniacal nitrogen (NH_3-N).

Variable	EE				HE				MON	CTL	SEM	Contrast *p*-Value					
	50	100	150	200	50	100	150	200				1	2	3	4	5	6
C2 (molar%)	71.58	70.50	72.00	67.77	72.11	70.47	71.08	69.59	66.52	63.05	2.58	0.22	0.06	**	0.80	0.15	0.49
C3 (molar%)	14.48	14.19	13.22	15.32	13.31	13.88	13.91	14.51	17.46	18.04	1.23	0.68	*	**	0.58	0.19	0.46
C4 (molar%)	9.19	10.00	9.62	10.95	8.87	9.86	9.48	10.33	8.24	10.04	0.79	0.03	0.01	0.65	0.36	0.03	0.60
C2:C3	5.00	5.01	5.52	4.55	4.88	5.11	4.50	4.82	3.82	3.76	0.43	0.87	*	**	0.40	0.31	0.052
SCFA (mM)	9.92	9.00	9.71	8.53	9.88	9.75	9.32	10.86	9.22	7.04	1.38	0.01	0.53	**	0.13	0.77	0.04
NH_3-N [A]	34.52	31.41	25.69	35.78	32.32	31.52	28.66	28.59	23.05	31.42	4.65	0.08	0.03	0.92	0.51	0.14	0.14

EE, ethanolic extract; HE, hydroalcoholic extract; EXT, grouping of EE and HE extracts in all doses; MON, 25 ppm monensin; CTL, control (no additive added); LVL, level effect; SEM, standard error of the mean. * $p < 0.01$, ** $p < 0.001$. [A] mg 100 mL^{-1}; C2, acetic acid; C3, propionic acid; C4, butyric acid; C2/C3, acetic/propionic ratio; SCFA, total short-chain fatty acids. Contrasts: 1, MON vs. CTL; 2, EXT vs. MON; 3, EXT vs. CTL; 4, EE vs. HE; 5, level effect; 6, effect of EXT vs. LVL interaction.

Regarding butyric acid (C4), despite not having a significant effect on the production of C2 and C3, the inclusion of monensin (MON) has a considerable impact. Monensin reduced C4 production by 18% compared to the CTL ($p = 0.03$) and by 15.83% compared to the EXT ($p = 0.01$). There was no difference ($p = 0.65$) between the EXT (9.79%) and the CTL (10.05%) for C4, but both treatments produced more C4 than MON (8.24%). The addition of the EXT levels had a significant effect ($p = 0.03$), resulting in higher concentrations for both the EE (10.95) and HE (10.33) at a 200 mL kg^{-1} DM level.

The total SCFA production was influenced by an interaction between the EXT and levels (LVLs) ($p = 0.04$), with HE (10.86 mM) providing higher SCFA production than EE (8.53 mM) at the 200 mL kg^{-1} DM level. Both the MON treatment (9.22 mM, $p = 0.015$) and the EXT (9.62 mM, $p = 0.0004$) provided a higher concentration of SCFA compared to the CTL (7.04 mM). The concentration of ammoniacal nitrogen (NH_3-N) was different ($p = 0.03$) between the EXT (31.06) and the MON (23.05).

3.3. Relative Microbial Quantification

The type of extract significantly ($p = 0.02$) affected the relative abundance of the *archaea*, with the EE (0.93) promoting a greater relative quantity of *archaea* compared to the HE (0.73) (Table 4). The relative abundance of *Fibrobacter succinogenes* showed a significant ($p = 0.02$) reduction of 33% between the EXT treatment (0.67) and the CTL (1.00).

Table 4. Effect of ethanolic (EE) and hydroalcoholic (HE) extracts on the relative population of microorganisms on rumen fluid.

	EE				HE				MON	CTL	SEM	Contrast *p*-Value					
Variable	**50**	**100**	**150**	**200**	**50**	**100**	**150**	**200**				**1**	**2**	**3**	**4**	**5**	**6**
Archaea	0.88	1.03	0.87	0.95	0.81	0.81	0.60	0.69	1.06	1.00	0.14	0.71	0.07	0.18	0.02	0.46	0.98
Fibrobacter succinogenes	0.67	0.68	0.60	0.52	0.84	0.78	0.74	0.52	0.67	1.00	0.21	0.07	0.96	0.02	0.26	0.26	0.57
Ruminococcus flavefaciens	1.03	1.10	0.95	1.08	0.71	0.84	0.78	0.79	1.21	1.00	0.13	0.20	0.02	0.46	*	0.54	0.59
Protozoa	1.13	0.82	1.13	0.51	1.62	1.36	0.99	1.49	0.66	1.00	0.29	0.35	0.09	0.62	0.01	0.81	0.04

EE, ethanolic extract; HE, hydroalcoholic extract; EXT, grouping of EE and HE extracts in all doses; MON, 25 ppm monensin; CTL, control (no additive added); LVL, level effect; SEM, standard error of the mean. * $p < 0.01$. Contrast: 1, MON vs. CTL; 2, EXT vs. MON; 3, EXT vs. CTL; 4, EE vs. HE; 5, level effect; 6, effect of EXT vs. LVL interaction.

The relative abundance of *Ruminococcus flavefaciens* had a significant effect ($p = 0.016$) when comparing the EXT treatment (0.91) with the MON (1.21) and a significant effect ($p = 0.005$) between the types of extracts, with the HE (0.78) showing a 25% decrease in the abundance of *R. flavefaciens* compared to the EE (1.04).

The relative abundance of protozoa in comparison to the control was only significant when comparing the EE and HE extracts ($p = 0.015$), with the EE (0.90) showing a lower relative quantity than the HE (1.37). There was also a significant interaction between the EXT and levels of inclusion ($p = 0.04$), with the HE (1.49) providing a higher abundance of protozoa than the EE (0.51) at the 200 mL kg^{-1} DM level.

4. Discussion

The study examined protodioscin from *Urochloa brizantha* extracts as a natural alternative to manipulate rumen microbial fermentation for methane reduction in sheep using an in vitro gas technique. We used *Urochloa brizantha* because it is the main source of feed for ruminants in Brazil and does not compete with human food sources. To our knowledge, there are no studies that have examined the production and use of *Urochloa brizantha* extracts to manipulate rumen fermentation.

As livestock, especially ruminants, contribute to global methane sources, several studies have investigated using plant extracts containing saponins as methane mitigators, utilizing the in vitro gas production technique [39–41]. In this study, including *Urochloa brizantha* extracts of 0.37 to 1.50 mg saponin (protodioscin) g^{-1} DM did not affect methane production and the mean value was 1.54 mL of CH4 g^{-1} DM. Similarly, Zhang et al. [6] evaluated increasing concentrations of "tea saponin" (0, 5, 10, 20 g kg^{-1}) in vitro and did not

obtain significant differences in the production of CH_4, with a mean 32.88 mL CH_4 g^{-1} DM. In the same way, Trotta et al. [16] found no effect on methane production in an in vitro trial testing increasing doses of *Y. schidigera* extracts (mL), despite having observed an increased linear ($p = 0.02$) gas production after 24 h with inclusion levels of YSE.

Nevertheless, Sahoo et al. [42] also used sheep inoculum donors in an in vitro experiment with fruit and vegetable waste and did not observe an effect on methane, however, when evaluating the CH_4 mL g^{-1} and DDM there was a significant reduction of 3.9 and 4.2% compared to a control. Niu et al. [17] also observed a 33.23% reduction in methane production ($p < 0.001$) when using fenugreek containing 10 to 12 µg mL^{-1} of steroidal saponin as a substrate compared to alfalfa. These discrepancies may be attributed to the different sources, types, and concentrations of saponin which can potentially alter responses in CH_4 production. Also, saponin levels generally correspond to increases or decreases in food degradability which has a direct influence on methane production. For example, the amount of saponin observed in fenugreek was 113.2 mg to a smilagenin equivalent of 100 g^{-1} [17], while in the Marandu grass extract, it was found to be a 3.62 mg protodioscin equivalent mL^{-1} in the EE and a 5.38 mg protodioscin equivalent mL^{-1} in the HE, which may have influenced the results observed. The substrate used can interact with secondary compounds that affect the rumen metabolic processes, potentially leading to synergetic or antagonistic effects on rumen fermentation [41].

Holtshausen et al. [43] and Freitas et al. [23] demonstrated that saponins, such as those from *Yucca schidigera* and *Urochloa humidicola*, respectively, can reduce methane emissions by decreasing concentrations of feed degradability. In our investigation, we did not observe a mitigating effect of steroid saponin on methane production, which was consistent with having no negative impact on feed degradability.

These discrepancies may be attributed to the lower saponin concentration in the *U. brizantha* extract used in our study. As suggested by Ebrahim et al. [44], lower saponin levels generally correspond to increased feed degradability, and a decrease in methane production is associated with a reduction in gas production due to a decrease in nutrient degradation [45]. Additionally, saponin can decrease degradability by reducing the fibrolytic enzyme activity in the rumen [46].

The partition factor (PF) is a relevant index linking the in vitro degradability of dry matter to gas production. According to Sallam et al. [47], a higher PF indicates a greater feed relative to gas production, suggesting an improvement in microbial protein synthesis and more efficient energy use by the microorganisms. Singh et al. [48], in their study on *Aloe vera* residue containing saponin, observed PF values ranging from 5.14 to 5.36 mg mL^{-1} at different concentrations (0, 10, 20, 30, and 40 g kg^{-1}). In our study, only the MON treatment showed a value (5.15 mg DMD mL^{-1}) near this. Blummel et al. [49] reported on PF ranges from 2.74 to 4.65 mg mL^{-1} for conventional roughages and cited that the change depends the type of substrate used. In our study using 70% corn silage, the average (4.87) remained near this parameter.

Regarding the impact of saponin on SCFA concentration, Trotta et al. [16] observed that the inclusion of *Y. schidigera* extract resulted in a linear reduction (1.90%) in the proportion of acetate, an increase (4.76%) in the proportion of propionate and, consequently, a reduction (6.97%) in the acetate: propionate ratio. Niu et al. [17] also observed more propionate (4.74%) and a decrease of 2.75% in the acetate: propionate ratio. Furthermore, some studies using extracts and plants containing steroidal saponins have reduced the molar proportion of butyrate [6,8,10].

In this study, we observed an increase in the molar ratios of acetate and butyrate, accompanied by a decrease in propionate. This outcome contradicts expectations regarding the predominant effects of saponins, which typically alter SCFAs by increasing the propionate and decreasing the acetate: propionate ratio. The shift was mainly attributed to their antiprotozoal effects which reduce acetate, a major product of protozoan degradation [13]. In addition, the propionate pathway is the only one that does not release carbon and hydrogen into the rumen. In this way, the methanogens cannot compete for hydrogen

because the hydrogen is utilized in the formation of propionate, which in turn reduces methane production [14]. Despite the observed increase in acetate, this change did not have any repercussions on methane production.

In general, we observed an increase in SCFA production, which is interesting because SCFAs account for 60% of the metabolizable energy for ruminants [36]. Another point is that the increase in SCFA production may be due to the partial degradation of the sugar portion of saponins by the microorganisms in the rumen [37].

As expected, the MON treatment was efficient in increasing propionate, reducing butyrate and decreasing NH_3-N, which could be associated with the efficiency of nitrogen use [39]. However, the EXT treatment showed no decrease in NH_3-N, which is associated with a constant protozoa population. Similarly, some studies using extracts and plants containing steroidal saponins did not observe this effect on ammonia nitrogen (NH_3-N) levels [9,11]. In contrast, other studies observed a reduction in in vitro ruminal fermentation with the increasing addition of a *Y. schidigera* extract [7,8]. According to Wina et al. [14], ruminal ammonia comes from two main sources, dietary degradation and microbial lysis, part of which is degraded by the rumen wall and the other is used directly by microorganisms.

Thus, steroidal saponins can indirectly affect ammonia nitrogen concentration by reducing the population of rumen-ciliated protozoa [38]. The reduction in ammonia nitrogen concentrations attributed to saponins is primarily due to their antiprotozoal effect, which limits nitrogen loss through predation by bacteria [50]. It is important to note that the presence of saponins does not always lead to a reduction in NH_3-N concentrations; in some cases, saponins may increase the bacterial population [51], or rumen microorganisms may adapt to their presence [52].

Several studies have found that saponins can modulate rumen microbial communities [7,40,45], as saponins can alter the extracellular surface tension of bacteria [53] by suppressing the population of protozoa and selectively inhibiting some bacteria [54]. The most predominant fibrolytic bacteria include *Fibrobacter succinogenes, Ruminococcus albus,* and *Ruminococcus flavefaciens*, with Fibrobacter species being gram-negative and Ruminococcus species being gram-positive [55]. According to Wang et al. [56], saponins from *Yucca* negatively affect gram-positive bacteria more than the gram-negative bacteria.

Goel, Makkar, and Becker [57] observed that *Sesbania saponin* reduced the population of protozoa and fungi while increasing population of bacteria such as *F. succinogenes* and *R. flavefaciens*. However, contrary to these findings, our study did not observe the expected antiprotozoal effect of saponin, which could have influenced the reduction in fibrolytic bacteria. Protozoa are known to engulf bacteria [58], and the absence of the expected reduction in protozoa in our study may have contributed to the differing outcomes regarding the fibrolytic bacteria. According to Hess et al. [51], saponin can decrease the methanogen population and methanogenic activity per unit of methanogenic cells, although this reduction does not always translate into a lower methane production. In our study, neither effect was observed in the methanogen population and methane production with use of the EXT. A reduction in *F. succinogenes*, a Gram-negative specialist cellulose degrading bacterium, was observed; however, acetate did not show a corresponding decrease.

Hu et al. [59] observed a 16% reduction in protozoan counts with the addition of 0.4 mg of tea saponins L^{-1} in rumen fluid. In contrast, Niu et al. [17] observed a 29.20% increase in the total bacteria and a 50.23% decrease in methanogen population when using fenugreek (rich in steroidal saponins) compared to alfalfa (rich in triterpene saponins). Additionally, Kim et al. [46] reported a reduction in ciliated protozoa counts with the administration of 37.26 and 45.65 mg g^{-1} DM of *Aloe saponaria* saponins. However, Hristov et al. [60], using the in vitro technique, observed that concentrations ranging from 44 to 176 mg L^{-1} of *Y. schidigera* and 100 to 400 mg L^{-1} of *Q. saponaria* did not influence the number of protozoa.

While many studies demonstrate a depressing effect of saponins on protozoal counts [14,47,57], some suggest that this antiprotozoal effect may be transient [14]. The inconsistencies among these findings highlight the complex effects of plant extracts and

their dosages. It is crucial to determine appropriate ranges for additive levels and further explore the specific properties of plant extracts in modulating rumen microorganisms [6].

5. Conclusions

The addition of *Urochloa brizantha* extracts to a sheep diet in vitro increased the production of acetate (C2) and short-chain fatty acids (SCFAs), with a decrease in *Fibrobacter succinogenes*, without affecting methane and the archaeal population. We did not produce results that could substantiate this extract being used as a feed additive, so further studies on the development of new extracts, the use of other techniques for extraction, and the dose optimization of *Urochloa brizantha* extracts are needed to substantiate practical applications in livestock management.

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