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Effect of Saponins from *Chenopodium quinoa* Willd. on Methane Production, Short Chain Fatty Acids and Digestibility *in vitro* Ruminal Fermentation

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ABSTRACT

The objective of this study was to assess the effects of saponins derived from *Chenopodium quinoa* by-products on methane (CH_4) production in a ruminal fermentation system under *in vitro* conditions. Additionally, the study included the analysis of short-chain fatty acids (SCFAs) and true dry matter digestibility (TDMD). The saponin extracts obtained from three quinoa varieties were Altiplano (AS), Quillahuaman (QS), and Salcedo (SS), and were tested at six different doses (0, 0.2, 0.4, 0.6, 0.8, and 1.0mg/mL) each. Rumen fluid was obtained from two fistulated Junín breed sheep, with alfalfa hay (AH) and a 1:1 mixture of maize and bran (MB) was used as basal substrates. The data were processed in a completely randomized design and replicated three times. The CH_4 per dry matter (mL/g DM), after 24h of incubation significantly decreased, acetate molar concentration decreased (p<0.05) with AS (3.6%) and QS (6.4%), propionate values increased (P<0.05) to 14.4% (AS), 15.7% (QS), and 15.5% (SS), the acetate-to-propionate ratio decreased (P<0.05) across all saponins treatments compared to the control and irrespective the substrates. The CH_4 to TDMD ratio decreased (P<0.05) with all saponins. Methane inhibition (mL CH_4/100mgTDMD) was higher by up 25.7% in the presence of AS, followed by QS at 18.7% and SS at 14.6%. The results indicate that saponins extracted from quinoa possess significant potential as feed additives for ruminants. Their application could contribute to the reduction of methane production, which would be advantageous for both livestock efficiency and environmental sustainability.

Key words: Extracts; Quinoa; CH_4; Ruminal fermentation; Substrates

INTRODUCTION

The increase in livestock production to meet the growing demand for human consumption poses a significant challenge due to its effects on global warming, specifically by emitting methane (CH_4) (Almeida et al. 2021; Kinley et al. 2021; Notarnicola et al. 2023). Enteric fermentation in ruminants (cattle, sheep, goats, camelids) is the primary source of this potent greenhouse gas and represents 46% of total CH_4 emissions in livestock farming (FAO 2023). In a global effort to reduce environmental impact and redirect the energy lost as CH_4, which can represent up to 12% of the gross energy ingested by ruminants (Öztürk and Gur 2021), various sustainable and natural mitigation strategies are being implemented (Tongwane and Moeletsi 2020; Balasundram et al. 2023). Among these strategies, the inclusion of plant secondary metabolites such as saponins in the diet of ruminants stands out as an alternative to synthetic substitutes (Ku-Vera et al. 2020; Tedeschi et al. 2021; Tyagi et al. 2022).

Saponins are a promising source of metabolites with the capacity to reduce CH_4 production (Jafari et al. 2019). These natural substances, present in various plants, have demonstrated their antimicrobial properties, which affect the microbial community in the rumen, including methanogenic archaea, the microorganisms responsible for CH_4 production (Kholif 2023). Numerous studies have explored the influence of saponins on methane production in both *in vitro* and *in vivo* settings (Canul-Solis et al. 2020; Dhanasekaran et al. 2020; Singh et al. 2020). For example, saponins extracted from *Tribulus terrestris* reduced CH_4 production in an *in vitro* fermentation experiment (Feng et al. 2012). It has also been reported that saponins can reduce CH_4 production and modify the profile of volatile fatty acids within the ruminal environment (Bodas et al. 2012). Furthermore, saponins from various plant sources, such as *Quillaja saponaria* and *Yucca schidigera*, have shown a significant reduction in CH_4 production, exceeding 60% at

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8mg/mL of saponins (Rira et al. 2015). One of the saponin-rich foods is quinoa, *Chenopodium quinoa* Willd. (El Hazzam et al. 2022), a pseudocereal of which Peru is the leading producer in the world. However, its byproduct, saponins, is underutilized. Saponins are bitter compounds located in the outermost layer of the seed, which serves a protective function (El Hazzam et al. 2020). Although saponins arise from a wide variety of quinoa, each with particular characteristics and primarily produced in the Andean regions, there is also increasing production in the coastal areas due to their favorable agroclimatic adaptability (Apaza et al. 2013). Moreover, these compounds exhibit antimicrobial properties (Kuljanabhagavad and Wink 2009; Sun et al. 2019; Dong et al. 2020). Nonetheless, only one *in vitro* study on ruminal fermentation examining the impact of quinoa-derived saponins on CH_4 generation at a specific inclusion level has been observed (Budan et al. 2013).

Due to the scarce studies regarding the consequence of saponins from various quinoa varieties on gas emissions, this study was planned to analyze the effects of saponins from by-products of three commercial quinoa varieties grown on the Peruvian coast, on methane production, short-chain fatty acids and diet digestibility in an *in vitro* ruminal fermentation system.

MATERIALS AND METHODS

Samples and extraction of quinoa saponins

Three Peruvian quinoa varieties were collected: INIA431-Altiplano, Salcedo-INIA and Quillahuaman-INIA, grown at 241 m.a.s.l. under conventional agronomic conditions at fertilizer levels of 250:150:120 kg NPK/ha in sandy-loam soils from the National Institute of Agricultural Innovation (INIA, acronym in *Spanish*) in La Molina, Lima (Perú) (12°5' S, 76°58' W). To obtain powdered saponin extract (Fig. 1), the Agro Market scarificator (Global-INIA, Peru) was utilized to remove the pericarp layer from quinoa seeds (El Hazzam et al. 2020). The by-product was collected in batches of 3kg from each quinoa variety, processed in triplicate. These were then mixed with distilled water at a 1:20 ratio and stirred for three hours. Subsequently, the mixture underwent a 24-hour maceration, followed by filtration using a vacuum pump (V-700® Buchi Labortechnik AG, Switzerland). The aqueous saponin extract was stored at 4°C, while the solids retained on the filter underwent a second extraction. All extracts were then dried in a tray dryer at 40°C and milled in a centrifugal mill (ZM-200® Retsch, Germany) (Guzmán et al. 2013).

Saponin content

Total saponin (Table 1) was determined by the UV-Vis spectrophotometric method (Lozano et al. 2012). In a tube, 0.5g of residual and 25mL of ethyl alcohol (50% v/v) were stirred in a shaker (M 37610-33® Thermo Scientific, USA) for 15 minutes. After macerating for one hour, the tubes were centrifuged at 4000rpm for 20 minutes at 25°C, and 5mL of the supernatant was collected. A calibration curve was constructed using standard oleanolic acid (Sigma Aldrich, USA) at concentrations of 100, 200, 300, 400, 500, and 600µg/mL. For the reaction, 1mL of each standard was mixed with 3.5mL of the Lieberman-Burchard reagent (a 1:5 ratio mixture of glacial acetic acid and sulfuric acid). Similarly, for the sample, 50µL of each extract and 950µL of a 50% v/v ethanolic solvent, along with the reagent, were combined. All tubes were vigorously vortexed for 30s and then allowed to stand for 40min. Absorbance at 528nm was measured using a Genesys 10S UV-Vis® spectrophotometer (Thermo Scientific, USA).

Table 1: Composition of substrates and total saponins in powdered extracts

Items (mg/g)	Substrates		Saponins extracts		
	AH	MB	AS	QS	SS
Dry matter	908.6	879	901.7	909.5	903.8
Crude protein	188.7	117.0	80.0	112.4	131
Crude fibre	183.0	34.3	0	1.2	0.4
Ether extract	11.6	20.2	0	1.9	0.8
Total saponins (mg oleanolic acid equivalents/g)	-	-	757.5	795.4	611.6

Nomenclature: AH=Alfalfa Hay; MB=Maize and bran mixture (1:1); solid saponin extracts: AS: Altiplano saponin; QS: Quillahuaman saponin and SS: Salcedo saponin.

In vitro ruminal fermentation and gas production

For *in vitro* ruminal fermentation, two substrates were used: AH (alfalfa hay) and MB (a 1:1 mixture of ground maize and wheat bran). Both basal materials were provided by the Nutrition Laboratory of Universidad Nacional Agraria La Molina, dried at 50°C for 48 hours, and ground in a mill (Thomas-Wiley®) with a 1mm screen. Dry matter, crude protein, ether extract, and crude fiber were analyzed in that substrates and saponin extracts according to Association of Analytical Communities (AOAC 2005) (Table 1).

The in vitro system was following the technique implemented by Theodorou et al. (1994) and modify by Mauricio et al. (2001). In bottles with rubber stoppers, 250mg of substrate, and saponin doses of 0, 0.2, 0.4, 0.6, 0.8, and 1.0mg/mL were used. Each saponin extract underwent twelve treatments (two substrates at six different doses each), performed in triplicate, plus three blanks containing only the incubation medium. The medium, prepared in a 5000mL flask, was by mixing 0.3mL of micromineral solution (13g of $CaCl_2.2H_2O$, 10g of $MnCl_2.4H_2O$, 1g of $CoCl_2.6H_2O$ and 8g of $FeCl_2.6H_2O$ were dissolved in 100mL of distilled water), 600mL of buffer solution (comprising 35g of $NaHCO_3$ and 4g of NH_4HCO_3 in 1000 distilled water), 600mL of macromineral solution (5.7g Na_2HPO_4, 6.2g KH_2PO_4 and 0.6g $MgSO_4.7H_2O$ in 1000mL of distilled water), 3mL of resazurin (0.1g of resazurin dissolved in 100mL distilled water) and 1200mL of distilled water. In addition, 120mL of reducing solution was added to the flask (625mg of $Na_2S.9H_2O$ and 625mg of L-Cysteine HCl, 4mL 1N NaOH in 100mL distilled water). The flask was placed on a magnetic stirrer at 39°C and infused with CO_2 for 90min to maintain the anaerobic environment.

The small ruminants housed at the Experimental Center for Sheep and Camelids of Universidad Nacional Agraria La Molina, Lima, Perú (12°5' S, 76°57' W) were fed twice a day consisting of a forage and concentrate mixture (2:1) and had unrestricted access to water. The rumen liquor (both solid and liquid components) was collected from various points in the lower middle zone of the rumen, four hours after the first morning feeding, from two fistulated Junin breed sheep. Following the management

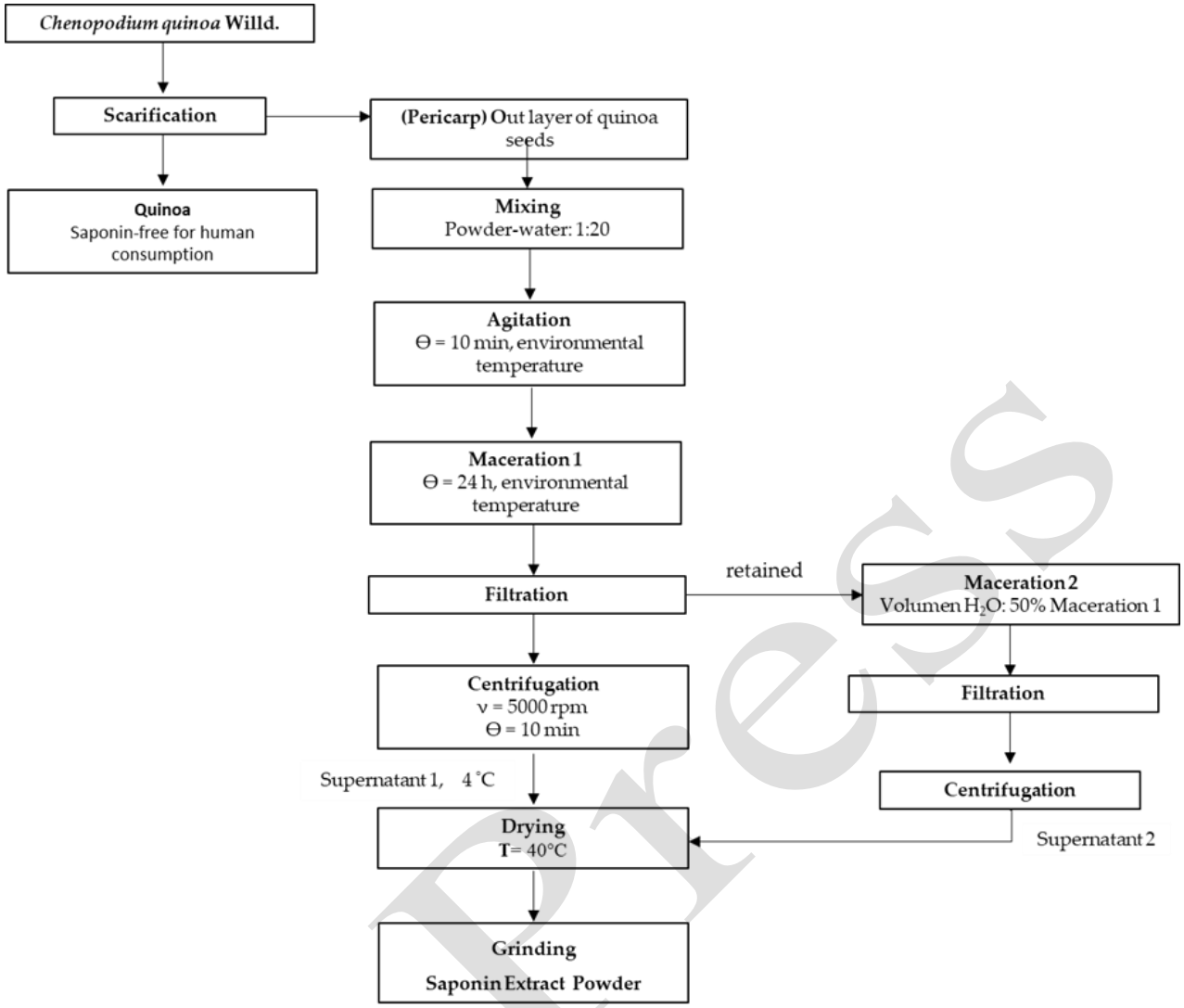

Fig. 1: Diagram followed to obtain powdered saponin extract from quinoa seeds.

and care guidelines in line with the Animal Care and Use Guidelines (Tucker et al. 2020) and Peruvian Law 30407 on principles of protection and animal welfare (Vega and Watanabe 2016).

The liquor was maintained at 39°C and passed through four stratums of muslin. The rumen liquid (600mL) mixed with the medium (2400mL) constituted the inoculum. Each treatment and blank were incubated with 25mL of inoculum and sealed with a rubber stopper followed by aluminum crimp. All fermentation processes were regulated by CO_2 gasification, and gas measurements were taken using a pressure transducer (JYB-KM®, Collihigh, China) connected to a 3-way stopcock. Gas output was recorded after 24h of incubation period in three runs separated by one week.

Determination of methane

The CH_4 concentration was measured from the gas in the headspace of the bottles by gas chromatography (Makkar and Vercoe 2007). A volume of 0.6mL of gas accumulated during 24h incubation was injected into an Agilent-USA 7890B® Gas Chromatography equipment employing a flame ionization detector (FID) and a GS-GASPRO capillary column of 30m x 320µm at 250°C temperature and He gas flow at 2.5 mL/min. A calibration curve was prepared using a CH_4 standard (99.99%) Praxair-USA. Time retention was 2.6min, and analysis time was 5 min. The CH_4 component readings were in ppm and the CH_4 production was expressed in mL of CH_4 per gram of dry matter.

True Dry Matter Digestibility (TDMD)

True dry matter digestibility (TDMD) was carried out after 24h incubation. The fermentation residue was recovered from the bottle and transferred into a filter bag (ANKOM, 25µm porosity size), which was sealed and treated with a neutral detergent solution using the fiber analyzer (Ankom Technology Corp., Macedon, NY) for one hour. Then, the bags were washed and dried at 105ºC for 24h. The loss of dry matter showed the degree degradation and the TDMD expressed in percentage (Van Soest et al. 1991).

Determination of Short Chain Fatty Acids (SCFAs)

The SCFAs were measured in the buffered rumen fluid after 24h of incubation by High Pressure Liquid Chromatography (HPLC) (Molina-Botero et al. 2020). Acetic, propionic and butyric acids were quantified by HPLC-Waters 2695 Separations Module (Waters, Milford-MA), using an Aminex HPX-87H cation exchange resin

column (300x7.8mm) equipped with an ion exclusion micro guard refill cartridge (Bio-Rad Laboratories Richmond-Ca, USA), a photodiode array detector (PAD), Waters 2996 and Empower software. Previously, each sample was filtered through #541 Whatman filter paper and centrifuged at 10000rpm for 10min. Then 2mL was collected in an Eppendorf tube and centrifuged again at 13000rpm for 2min. The supernatant was passed through a 0.22µm Millipore filter, type GV (Millipore, Bedford). The chromatographic conditions were a mobile phase of 0.005M sulfuric acid, a volumetric flow rate of 0.6mL/min at 50ºC and a dilution factor of ten. SCFAs were measured by comparing acetic, propionic and butyric acid standards with time retention of 15.053, 17.710 and 21.663min, respectively at 210nm.

Statistical analysis

The IBM SPSS Statistics Software Version 22 was applied to evaluate the effect of substrates and different levels of saponins on gas production, CH_4, SCFAs and TDMD. The data were analyzed using Analysis of Variance (ANOVA) through the General Linear Model (GLM) procedure in SPSS, employing a completely randomized design with a factorial arrangement of 2 substrates x 6 inclusion levels, as detailed below:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \xi_{ijk}$$

where:

Y_{ijk}: is the response variable of the i^{th} substrate and the j^{th} level of saponin inclusion.

μ: is the general average

α_i: is the effect of the i^{th} substrate (i=1 or 2)

β_j: is the effect of the j^{th} level of saponin inclusion (j= 1.2, .6)

$(\alpha\times\beta)_{ij}$: is the effect of the interaction between the i^{th} substrate and the j^{th} level of saponin inclusion

ε_{ijk}: is the experimental error

The Tukey test was applied for multiple comparison testing between treatments with significant differences were identified at $p<0.05$, and a trend towards significance was noted at $0.05\leq p<0.10$.

RESULTS

Gas production and True Dry Matter Digestibility (TDMD)

The gas produced after 24h of *in vitro* incubation is presented in Table 2. The substrates, irrespective of the saponin inclusion level, significantly (P<0.05) affected gas production. The MB substrate generated higher gas production (276.94mL/g DM on average) than AH substrate (191.20mL/g DM on average). Total gas production was not significantly affected ($p>0.05$) by dose variation across all treatments. On the other hand, the percentage of TDMD did not vary (P>0.05) with the addition of low doses (0.2-0.6mg/mL) of AS and QS extracts, regardless of substrates. However, at higher doses as 0.8mg/mL, the TDMD percentage increased ($p<0.05$) compared to the control and the rest of the treatments. For the SS extract, none of the doses had a significant effect (P>0.05) on total gas production.

Short Chain Fatty Acids (SCFAs)

As reported in Table 3, the molar concentrations of acetic acid in an *in vitro* fermentation after 24 hours of incubation exhibit a trend (P=0.067) in the AS interaction, showing a slight decrease at higher doses (0.8mg/mL) with the AH substrate compared to the control. No significant effect (P>0.05) was reported in SS, but in QS with the AH substrate at the highest inclusion level, a lower molar concentration of acetate is observed compared to the MB substrate at the same level. Moreover, the incorporation of quinoa saponins, irrespective of substrates, significantly reduced the acetate proportion at concentrations exceeding 0.4mg/mL by 3.6% with AS and by 6.4% at the maximum

Table 2: Effect of saponin extracts of Altiplano (AS), Quillahuaman (QS) and Salcedo (SS) on Gas production (mL/g DM) and True Dry Matter Digestibility (TDMD) in percentage

Extract	Substrate	Saponin extract level(mg/mL)						Mean of substrate	SEM	P value		
		0	0.2	0.4	0.6	0.8	1			Substrate	Level	Interaction
Gas Production (mL/g DM)												
AS	AH	189^{b}	197^{b}	192^{b}	187^{b}	188^{b}	186^{b}	189.7	4.36	<0.0001	0.547	0.352
	MB	279^{a}	274^{a}	275^{a}	270^{a}	274^{a}	280^{a}	275.4				
Mean of levels		234	235	233	228	231	233					
QS	AH	189^{b}	196^{b}	189^{b}	196^{b}	192^{b}	182^{b}	190.8	4.35	<0.0001	0.191	0.211
	MB	279^{a}	275^{a}	274^{a}	275^{a}	277^{a}	275^{a}	275.8				
Mean of levels		234	236	232	235	234	228					
SS	AH	189^{b}	195^{b}	199^{b}	190^{b}	195^{b}	191^{b}	193.2	4.43	<0.0001	0.299	0.142
	MB	279^{a}	273^{a}	278^{a}	283^{a}	286^{a}	278.0	279.6				
Mean of levels		234	234	239	236	240	234					
TDMD (%)												
AS	AH	64.5^{c}	65.7^{c}	64.7^{c}	66.5^{c}	66.3^{c}	66.1^{c}	65.6	1.45	<0.0001	0.009	0.114
	MB	80.5^{b}	79.9^{b}	82.5^{ab}	82.0^{ab}	85.6^{a}	83.7^{ab}	82.4				
Mean of levels		72.5^{B}	72.8^{B}	73.6^{AB}	74.3^{AB}	75.9^{A}	74.9^{AB}					
QS	AH	64.5^{b}	67.8^{b}	68.6^{b}	64.6^{b}	68.6^{b}	67.8^{b}	66.9	1.12	<0.0001	0.025	0.321
	MB	80.5^{a}	81.7^{a}	81.5^{a}	81.4^{a}	82.6^{a}	81.3^{a}	81.5				
Mean of levels		72.5^{B}	74.7^{AB}	75.1^{AB}	72.9^{AB}	75.6^{A}	74.5^{AB}					
SS	AH	64.5^{b}	63.0^{b}	64.2^{b}	66.6^{b}	63.4^{b}	64.6^{b}	64.4	1.57	<0.0001	0.457	0.598
	MB	80.5^{a}	81.2^{a}	80.1^{a}	82.1^{a}	81.6^{a}	78.6^{a}	80.7				
Mean of levels		72.5^{A}	72.1^{A}	72.2^{A}	74.4	72.5^{A}	71.6^{A}					

Abbreviations: AH: Alfalfa hay; MB: Maize-bran mixture. Means not sharing upper-case letters are significantly different ($p<0.05$) among saponins levels, irrespective of substrates. Means not sharing lower-case letters differ significantly ($p<0.05$) among saponins levels and substrates. SEM: Standard error of the mean.

Table 3: Effect of saponins concentrates Altiplano (AS), Quillahuaman (QS) and Salcedo (SS) on the molar concentration of Acetic and Propionic acids (mM) after 24 h of incubation

Extract	Substrate	Level of saponin (mg/mL)						Mean of substrate		*p*-value		
		0	0.2	0.4	0.6	0.8	1.0		SEM	Substrate	Level	Interaction
Acetic acid (mmol/L)												
AS	AH	41.96^{a}	41.15^{ab}	41.06^{ab}	40.31^{abc}	40.13^{bc}	40.39^{abc}	40.83	0.16	<0.0001	0.003	0.067
	MB	40.61^{abc}	39.73^{bc}	38.91^{c}	39.68^{bc}	40.18^{bc}	39.21^{c}	39.72				
	Mean of levels	41.28^{A}	40.44^{AB}	39.98^{B}	39.99^{B}	40.15^{B}	39.80^{B}					
QS	AH	41.96^{a}	41.15^{a}	40.80^{a}	41.28^{a}	40.08^{a}	35.77^{b}	40.17	0.3	0.002	<0.0001	<0.0001
	MB	40.61^{a}	42.60^{a}	40.30^{a}	41.47^{a}	40.66^{a}	41.50^{a}	41.19				
Mean of levels		41.28^{A}	41.87^{A}	40.55^{A}	41.37^{A}	40.37^{A}	38.63^{B}					
SS	AH	41.96^{a}	41.69^{ab}	40.98^{ab}	41.93^{a}	41.53^{ab}	40.78^{ab}	41.48	0.14	0.001	0.024	0.737
	MB	40.61^{ab}	41.38^{a}	38.83^{b}	40.88^{a}	40.70^{ab}	40.25^{ab}	40.61				
Mean of levels		41.28^{A}	41.53^{A}	40.41^{A}	41.41^{A}	41.11^{A}	40.51^{A}					
Propionic acid (mmol/L)												
AS	AH	14.42^{e}	14.22^{e}	14.41^{e}	14.84^{e}	15.22^{e}	14.71^{e}	14.63	0.53	<0.0001	<0.0001	<0.0001
	MB	18.42^{d}	18.95^{cd}	20.50^{bc}	20.40^{c}	22.34^{a}	22.08^{ab}	20.45				
Mean of levels		16.42^{D}	16.58^{CD}	17.46^{C}	17.62^{BC}	18.78^{A}	18.39^{AB}					
QS	AH	14.42^{d}	14.69^{d}	14.85^{d}	14.88^{d}	15.07^{d}	13.74^{d}	14.61	0.6	<0.0001	<0.0001	<0.0001
	MB	18.42^{c}	20.19^{b}	20.44^{b}	23.10^{a}	22.86^{a}	23.39^{a}	21.40				
Mean of levels		16.42^{D}	17.44^{CD}	17.64^{BC}	18.99^{A}	18.96^{A}	18.56^{AB}					
SS	AH	14.42^{gh}	14.34^{h}	15.69^{ef}	16.21^{e}	15.14^{fgh}	15.22^{fg}	15.17	0.54	<0.0001	<0.0001	0.001
	MB	18.42^{d}	19.98^{c}	20.17^{c}	21.47^{b}	22.78^{a}	22.49^{a}	20.88				
Mean of levels		16.42^{D}	17.16^{C}	17.93^{B}	18.84^{A}	18.96^{A}	18.85^{A}					

Abbreviations: AH: Alfalfa hay; MB: Maize-bran mixture. Means not sharing upper-case letters are significantly different (p<0.05) among saponins levels, irrespective of substrates. Means not sharing lower-case letters differ significantly (P<0.05) among saponins levels and substrates. SEM: Standard error of the mean.

Table 4: Effect of saponins concentrates Altiplano (AS), Quillahuaman (QS) and Salcedo (SS) on A/P proportions and Butyric acid (mM) after 24 h of incubation

Extract	Substrate	Level of saponins (mg/mL)						Mean of substrate	*p*-value			
		0	0.2	0.4	0.6	0.8	1		SEM	Substrate	Level	Interaction
A/P												
AS	AH	2.91^{a}	2.90^{a}	2.85^{ab}	2.72^{bc}	2.64^{c}	2.75^{bc}	2.79	0.075	<0.0001	<0.0001	<0.0001
	MB	2.21^{d}	2.10^{d}	1.90^{ef}	1.95^{e}	1.80^{f}	1.79^{f}	1.95				
Mean of levels		2.56^{A}	2.50^{A}	2.37^{B}	2.33^{BC}	2.22^{D}	2.26^{CD}					
QS	AH	2.91^{a}	2.80^{ab}	2.75^{abc}	2.78^{abc}	2.66^{bc}	2.60^{c}	2.75	0.073	<0.0001	<0.0001	0.007
	MB	2.21^{d}	2.11^{de}	1.98^{ef}	1.80^{fg}	1.78^{g}	1.77^{g}	1.94				
Mean of levels		2.56^{A}	2.46^{AB}	2.36^{BC}	2.29^{CD}	2.22^{D}	2.19^{D}					
SS	AH	2.91^{a}	2.91^{a}	2.61^{bc}	2.59^{c}	2.74^{b}	2.68^{bc}	2.74	0.071	<0.0001	<0.0001	0.122
	MB	2.21^{d}	2.07^{de}	1.97^{ef}	1.91^{fg}	1.79^{g}	1.79^{g}	1.96				
Mean of levels		2.56^{A}	2.49^{A}	2.29^{B}	2.25^{B}	2.27^{B}	2.23^{B}					
Butyric acid (mmol/L)												
AS	AH	3.41^{b}	3.61^{b}	3.67^{b}	3.76^{b}	3.74^{b}	3.75^{b}	3.65	0.47	<0.0001	<0.0001	<0.0001
	MB	9.34^{a}	9.70^{a}	10.13^{a}	8.58^{a}	8.07^{a}	8.75^{a}	9.09				
Mean of levels		6.37^{ABC}	6.65^{AB}	6.90^{A}	6.17^{BC}	5.90^{C}	6.25^{BC}					
QS	AH	3.41^{d}	3.70^{d}	3.71^{d}	3.77^{d}	3.91^{d}	3.36^{d}	3.64	0.42	<0.0001	<0.0001	<0.0001
	MB	9.34^{a}	9.70^{a}	8.03^{bc}	8.54^{b}	7.60^{c}	7.76^{c}	8.49				
Mean of levels		6.37^{AB}	6.70^{A}	5.87^{CD}	6.16^{BC}	5.76^{CD}	5.56^{D}					
SS	AH	3.41^{c}	3.32^{c}	3.36^{c}	3.42^{c}	3.57^{c}	3.50^{c}	3.43	0.49	<0.0001	<0.0001	0.001
	MB	9.34^{a}	8.10^{b}	9.51^{a}	9.58^{a}	9.06^{a}	9.71^{a}	9.21				
Mean of levels		6.37^{A}	5.71^{B}	6.43^{A}	6.50^{A}	6.31^{A}	6.60^{A}					

Abbreviations: AH: Alfalfa hay; MB: Maize-bran mixture. Means not sharing upper-case letters are significantly different (p<0.05) among saponins levels, irrespective of substrates. Means not sharing lower-case letters differ significantly (p<0.05) among saponins levels and substrates. SEM: Standard error of the mean.

inclusion level with QS compared to control. All studied saponins increased (P<0.05) the proportion of propionate across all doses. Saponins at higher levels exhibited better yields, inclusions above of 0.8mgAS/mL by 14.3%, 0.6mg QS/mL by 15.7% and 0.6mg SS/mL by 15.5% compared to the control. The proportion of propionate in the MB substrate showed a significant increase (P<0.05) compared to the AH substrate across all treatments. Additionally, the interaction between AH substrate and inclusion levels revealed lower molar concentrations of propionate compared to MB.

The results of the acetate to propionate (A/P) ratio are presented in Table 4. At doses higher than 0.4mg/mL, irrespective of substrates, the A/P ratio decreases (P<0.05) significantly in all varieties of saponins. The A/P values are significantly (p>0.05) higher in AH substrate compared to MB. The interactions (P<0.05) between saponins doses and substrates are shown not only when comparing quantities within the same substrate but also between substrates. At higher doses of *Chenopodium quinoa* saponin extracts, acetate/propionate ratio decreased by up to 13.3% (0.8mgAS/mL), 14.5% (1.0mgQS/mL) and 12.9% (1.0mgSS/mL) compared with the control. Further, the Table 4, shows the effects of different levels of AS, QS, and SS on the molar concentration of butyric acid. The

addition of quinoa saponin extracts, irrespective of the substrate, did not vary (p>0.05) the butyrate concentrations at lower doses of 0.2-0.4mg/mL AS, 0.2mg/mL QS, and at all doses of SS except lower dose. The remaining doses significantly decreased (P<0.05) with the inclusion of quinoa saponins after 24 hours of incubation.

Methane

The CH_4 production after 24h *in vitro* incubation is shown in Table 5. The results reveal the significant effect (P<0.05) of saponins inclusion on CH_4 emissions in rumen fermentation. The inclusion of AS at 0.8 and 1.0mg/mL, irrespective of the substrate, presents similar effects (p>0.05), but both significantly reduced CH_4 production values compared to lower inclusions and the control (without saponin) achieving a maximum CH_4 reduction of 22.34%. While with QS at an inclusion level of 0.6mg/mL, methane production decreased by up to 19.98% compared to the control, with values at higher levels being statistically similar to each other, but all lower than the control. About SS variety inclusions, levels higher than 0.8mg/mL resulted in significantly reduced CH_4 production values, with a CH_4 inhibition of up to 14.92%. A significant reduction (P<0.05) in the CH_4 to true digestibility ratio (CH_4 mL/100mg TDMD) was observed with varying saponin levels after 24 hours of incubation (Fig. 3). The study further demonstrated that higher doses of quinoa saponin extracts led to a decrease in the CH_4 to TDMD ratio by up to 25.7% (0.8mg AS/mL), 18.9% (0.6mg QS/mL) and 12.3% (1.0mg SS/mL). Moreover, significant differences (P<0.05) were found between the AH and MB substrates, with average values of 4.53 and 6.47mL CH_4/100mg TDMD, respectively, irrespective of saponin levels.

Table 5: Effect of saponin extracts of Altiplano (AS), Quillahuaman (QS) and Salcedo (SS) on methane production in mL CH_4/g DM and the proportion of methane production to TDMD (mL CH_4/100 mgTDMD) after 24 h of incubation

Extract Substrate		Level of saponins (mg/mL)						Mean of Substrate	SEM	*p*-value		
		0	0.2	0.4	0.6	0.8	1			Substrate	Level	Interaction
CH_4 Production (mL/g DM)												
AS	AH	31.1^{de}	32.9^{cde}	29.6^{e}	28.0^{e}	25.6^{e}	28.5^{e}	29.3	2.06	<0.0001	0.001	0.211
	MB	57.3^{a}	57.9^{a}	54.3^{ab}	48.1^{b}	43.1^{bcd}	44.0^{bc}	50.8				
Mean of levels		44.2^{A}	45.4^{A}	42.0^{AB}	38.1^{AB}	34.3^{B}	36.3^{B}					
QS	AH	31.1^{bc}	33.4^{b}	29.5^{bc}	21.8^{c}	28.7^{bc}	26.6^{bc}	28.5	2.21	<0.0001	0.0003	0.471
	MB	57.3^{a}	54.9^{a}	50.9^{a}	48.9^{a}	48.0^{a}	50.3^{a}	51.7				
Mean of levels		44.2^{A}	44.1^{A}	40.2^{AB}	35.4^{B}	38.3^{AB}	38.4^{AB}					
SS	AH	31.1^{d}	32.8^{d}	28.9^{d}	27.6^{d}	30.0^{d}	27.6^{d}	29.7	2.06	<0.0001	0.001	0.285
	MB	57.3^{a}	56.6^{a}	55.3^{ab}	53.0^{abc}	49.6^{bc}	47.8^{c}	53.3				
Mean of levels		44.2^{AB}	44.7^{A}	42.1^{ABC}	40.3^{BCD}	39.8^{CD}	37.7^{D}					
CH_4 (mL/100mg TDMD)												
AS	AH	4.9^{cd}	5.1^{bcd}	4.6^{cd}	4.3^{d}	3.9^{d}	4.4^{cd}	4.5	0.20	<0.0001	<0.0001	0.194
	MB	7.2^{a}	7.3^{a}	6.6^{ab}	5.9^{abc}	5.1^{bcd}	5.3^{bcd}	6.2				
Mean of levels		6.0^{AB}	6.2^{A}	5.6^{ABC}	5.1^{BCD}	4.5^{D}	4.9^{CD}					
QS	AH	4.9^{bc}	5.0^{bc}	4.3^{c}	3.6^{c}	4.3^{c}	3.9^{c}	4.3	0.21	<0.0001	0.002	0.584
	MB	7.2^{a}	6.8^{a}	6.3^{ab}	6.2^{ab}	5.9^{ab}	6.1^{ab}	6.4				
Mean of levels		6.0^{A}	5.9^{AB}	5.3^{ABC}	4.9^{C}	5.1^{BC}	5.0^{BC}					
SS	AH	4.9^{d}	5.3^{bcd}	4.7^{d}	4.3^{d}	4.9^{cd}	4.5^{d}	4.8	0.19	<0.0001	0.013	0.146
	MB	7.2^{a}	7.0^{a}	7.1^{a}	6.8^{a}	6.4^{ab}	6.1^{abc}	6.8				
Mean of levels		6.0^{A}	6.1^{A}	5.9^{AB}	5.6^{AB}	5.7^{AB}	5.3^{B}					

Abbreviations: AH: Alfalfa hay; MB: Maize-bran mixture. Means not sharing upper-case letters are significantly different (p<0.05) among saponins levels, irrespective of substrates. Means not sharing lower-case letters differ significantly (p<0.05) among saponins levels and substrates. SEM: Standard error of the mean.

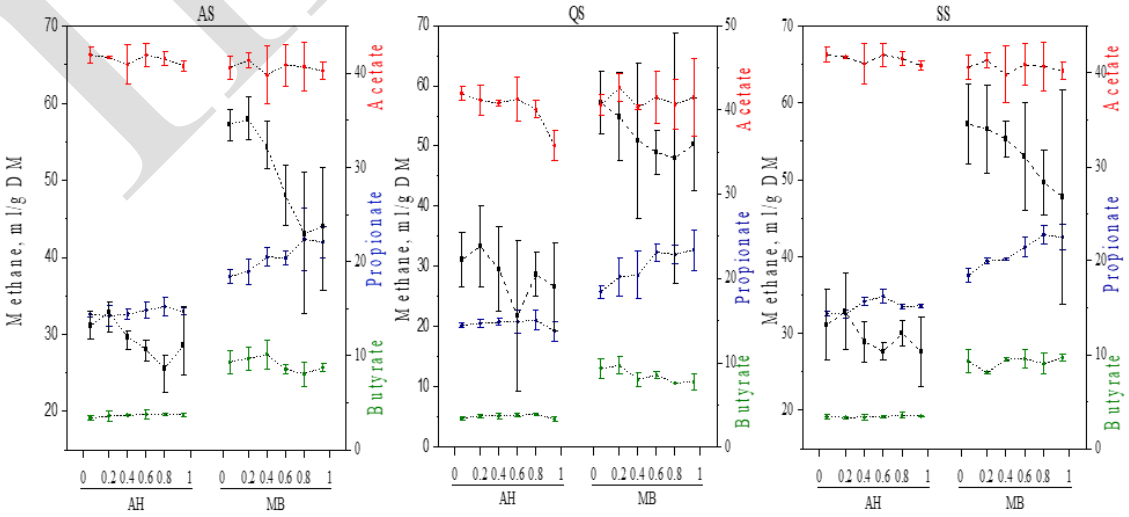

Fig. 2: Effects of short-chain fatty acids (acetic, propionic and butyric) in mmol/L and methane production in mL/g DM of alfalfa hay (AH) and maize-bran mixture (MB) on an addition of increasing doses of quinoa saponins: Altiplano (AS), Quillahuaman (QS) and Salcedo (SS).

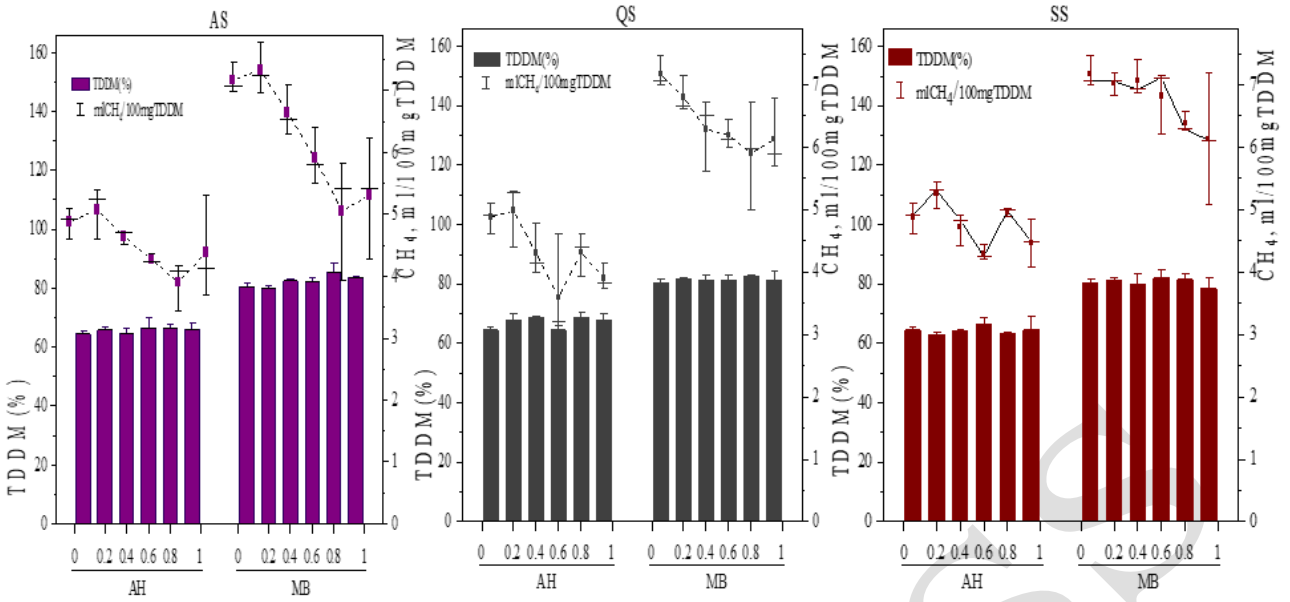

Fig. 3: Comparison of TDMD (%) and CH_4 to TDMD ratio (mL/100mg TDMD) in the AS, QS and SS saponin concentrates from *Chenopodium quinoa* at different inclusion levels containing AH and MB substrates.

DISCUSSION

Gas production and True Dry Matter Digestibility (TDMD)

Previous studies reported that saponins-rich plant extracts as feed additives in high-concentrate diets, with higher levels of non-fiber carbohydrates such as starch, increased gas production compared to a diet predominantly composed of forage (Jayanegara et al. 2020). For instance, the inclusion of *Camellia sinensis* saponin extracts in varying forage-to-concentrate ratios as substrates, demonstrated that higher gas production was achieved at a ratio of 30:70 (49.9 mL/200mg) compared to 70:30 (44.6mL/200mg) (Jadhav et al. 2018).

The gas produced during fermentation depends on the availability of carbohydrates, with lipids and proteins being less fermentable (Aderao et al. 2018). This can explain the higher gas production in the MB mixture compared to AH, as well as the non-significant effect (p>0.05) on gas production when associated with quinoa saponin aqueous extracts of varying levels. Some triterpene saponins have the same behavior (Canul-Solis et al. 2020; Unnawong et al. 2021). Similarly, gas production did not vary when comparing 0.6mg/mL of saponins from the aqueous extract of *Yucca schidigera* in hay with the control (Makkar et al. 1998).

In relationship with digestibility, these results are consistent with the inclusion of purified saponins at a level of 0.21mg/mL, which did not affect TDMD (Bharathidhasan et al. 2013), suggesting that the saponins contained can improve nutrient degradability by increasing the microbial population as bacteria, protozoa and fungi zoospore (Matra et al. 2021). However, in the *in vitro* fermentation studies conducted by Makkar et al. (1998), it was reported that the addition of saponins resulted in a reduction in gas and SCFA production, while the extent of the truly degraded substrate remained unchanged or increased.

Short Chain Fatty Acids (SCFAs)

The decrease in the molar concentration of acetate as saponin inclusion increases is consistent with the behavior of other similar saponins. For instance, at 0.9mg/mL, saponins from *Tribulus terrestris* were found to decrease acetate (Feng et al. 2012) and aqueous and ethanolic extracts of saponins from *Sapindus mukorosii* showed lower acetate concentrations at higher doses (Singh et al. 2020). Conversely, other *in vitro* studies using purified saponins at various levels (ranging from 1.55 to 6.20mg/30mL of inoculum) reported unaltered molar concentrations of acetic acid as observed with SS (Bharathidhasan et al. 2013). Similar studies involving saponin extracts, such as *Sesbania grandiflora* pod saponins (Unnawong et al. 2021), tea saponins (Liu et al. 2019) and *Tribulus terrestris* saponins (Feng et al. 2012), have also documented an increased proportion of propionate in an *in vitro* rumen fermentation study. Lower molar acetate and higher propionate molar concentrations could mean that the addition of saponins caused inhibitory effects on protozoa (Singh et al. 2020) and a shift in hydrogen direction from methanogenesis towards propionate production (Patra and Saxeda 2010; Rira et al. 2015).

Similar *in vitro* studies described a linear reduction in the acetate to propionate ratio for *Quillaja saponins* and *Gypsophilla saponins* (Castro-Montoya et al. 2011) that may be because of amphiphilic structure of saponins (Fleck et al. 2019). Some studies indicate concentration of butyrate using saponins extracts of *Sesbania grandiflora* decreased (Unnawong et al. 2021) and *Quillaja saponins* tend to decrease (Castro-Montoya et al. 2011). On the contrary, other researchers reported that when adding doses of saponins the molar concentration of butyrate remains similar in all their treatments (Patra et al. 2006). Two ways to explain the decrease in CH_4 are suggested, either there is a reduction in methanogen populations, which means a reduction in protozoa numbers (Singh et al. 2020), or there is a direct inhibitory effect on methanogenic archaea by

Chenopodium quinoa saponins, similar to the effect of *Sapindus saponaria* saponins (Patra and Saxena 2010). In general, the SCFAs values, using MB substrate are higher than those of AH, resulting in greater CH_4 emissions (Fig. 2). The more soluble fraction contained in MB against AH could mean greater fermentable carbohydrates available for rumen microbes, which could lead to faster production of fatty acids (Matra et al. 2021).

Methane

Budan et al. (2013) investigated the effect of *Chenopodium quinoa* hulls on methane production using a 70:30 ratio of dry ryegrass forage and wheat seeds, but their experiment was conducted at only one dose (0.4mg/mL), which did not result in significant differences compared to the control. Similarly, this study observed no significant effects at the same dose, but it was evident that quinoa saponins demonstrated a positive effect on methane mitigation at higher doses. Additionally, the literature reports on other natural sources of saponins, particularly those with triterpene structures that have been shown to inhibit CH_4 production. For example, 0.4mg/mL of saponins from *Camellia sinensis* reduced CH_4 production by 8% (Guo et al. 2008), 2mg/mL of saponins from *Sapindus rarak* DC inhibited CH_4 production by 4.6% to 16.2% (Jayanegara et al. 2020) and 120mg/g of crude saponins from *Sapindus saponaria* L. led to a 20% reduction in CH_4 compared to the control (Hess et al. 2003).

Many studies on CH_4 in ruminants point out that pure saponins or saponin extracts could have an inhibitory effect on CH_4 production, probably due to suppression of protozoan population and presumably the reduction of methanogen activity (Guo et al. 2008; Jadhav et al. 2018). Similarly, it was observed that the decrease in rumen CH_4 due to the inhibition of methanogens is attributed to the reduction of H_2, leading to a lower concentration of acetate and a higher concentration of propionate (Li et al. 2024). Fermentation that produces acetate and butyrate generates more hydrogen, providing a substrate for methanogenic archaea that reduce CO_2 to produce CH_4 (Moss et al. 2000). This work obtained similar results to those observed with purified *Quillaja saponaria* saponins, which decreased by 21% (1.25mg/mL) (Castro-Montoya et al. 2011), suggesting that feed intake is an important predictor of methane emissions (Congio et al. 2022).

On the other hand, other researchers have indicated that increased digestibility leads to higher methane production per unit of DM intake. Furthermore, the observed methane emissions and nutrient composition of these substrates are consistent with studies reporting that a greater amount of fermentable carbohydrates is positively associated with CH_4 emissions. In contrast, lower fiber digestibility and the lack of impact from ether extract are negatively associated with methane emissions, primarily due to the respective increase or decrease in the hydrogen requirements by methanogenic microorganisms (Patra et al. 2016).

The reduction in CH_4 observed with the *in vitro* addition of aqueous quinoa saponin extracts is consistent with studies on other saponin sources, such as aqueous extracts of *Sapindus mukorossi* (Agarwal et al. 2006). Differences in methane emissions and other parameters were observed among varieties of *Chenopodium quinoa* seeds, particularly with the SS extract. The saponin content in the powdered extract is influenced by the genetic diversity of quinoa, characterized by its wide range of accessions. This genetic diversity is a focus of research aimed at improving agro-industrial production, as demonstrated by the quinoa varieties used in this study (Apaza et al. 2013). The antimethanogenic response of these secondary metabolites is shaped by their genetic composition, which affects their antimicrobial activities and nutritional profiles, both of which are dependent on the plant's production and cultivation cycles (Rojas et al. 2016; Dong et al. 2020).

Conclusion

The saponin extracts from *Chenopodium quinoa* by-products are potential inhibitors of methane emissions in an *in vitro* ruminal fermentation as feed additives, irrespective of substrates. According to the results, better antimethanogenic activity occurs with AS at 0.8mg/mL, that could modulate the fermentation parameters like SCFAs as well as the TDMD in favor of methane reduction. Beyond this, it is recommended to evaluate the ruminal microbial mass to complement the biological characterization of saponins in substrates before *in vivo* studies. Finally, the saponins extracts applied in this study are free of organic solvents, contributing not only to the reduction of greenhouse gases and the improvement of animal production systems but also to the valorization of solid residues.

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