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Evaluation of in vitro energy distribution and methanogenic potential of two forages with the addition of condensed tannins

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ABSTRACT. The objective of this work was to analyze the effect of the addition of condensed tannins (CT) in the efficiency of digestion, methanogenic potential and energy distribution between the fermentation products of two forages. An assay was carried out using the *in vitro* gas production technique in which extracts of Quebracho (*Schinopsis balansae*) and *Lotus corniculatus* were evaluated with fermentation patterns of derived products from Ryegrass (RG, *Lolium perenne*) and a tropical forage, *Megathyrsus maximus* (MM). Tannins were added to the substrate at a concentration of 30 mg g^{-1}. MM presented higher and delayed gas production (GP), and *in vitro* dry matter, organic matter and fiber digestibilities (ivDMD, ivOMD and NDFD, respectively) were relatively high but lower than RG. In addition, MM presented higher CH_4 production (CH_4p) than RG in 24 and 48h. Even though CT of Quebracho induced a decrease in the NDFD, contrary to what was expected, CH_4p was greater, although this effect could not be attributed to the presence of CT. The stoichiometric evaluation indicated that while the highest CH_4p in Quebracho treatments were associated with acetogenic profiles, CH_4p with Lotus did not show any relationship with the volatile fatty acids (VFA) profile, but it did show a relationship with the highest total VFA production and the highest GP.

Keywords: secondary compounds; methane production; *in vitro* gas production; ryegrass; *Megathyrsus maximus*.

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Introduction

Greenhouse gases generated by ruminants (approximately 7.1 Gt of CO_2-eq $year^{-1}$) are environmental pollutants, of which methane (CH_4), the most related to human activity, represented a significant feed energy loss of about 5-7% of dietary gross energy (Hristov et al., 2013). Considering the environmental and energetic importance of CH_4 production (CH_4p), numerous mitigation programs such as diet strategies have been performed. However, further research on mitigation techniques is required (Kumar et al., 2014).

Condensed tannins (CT) have been studied as additives in diets for ruminants. Although they may have undesirable effects (*e.g.* they decrease voluntary intake, reduce diet digestibility, Abd El Taw & Khattab, 2018) some extracts, in the correct dose, may improve the use of some nutrients (*e.g.* reducing ruminal protein degradability allowing their use in later portions of the gastrointestinal tract, Patra & Saxena, 2011). On the other hand, CT have been proposed as additives in forage-based diets for their antimicrobial action on methanogenic *archaea* (Min & Solaiman, 2018). The relationship between feed degradability and fermentation-derived products can be explained by means of stoichiometric studies as described by Wolin (1960), relating accumulation of gas and volatile fatty acids (VFA). Likewise, the synthesis of microbial biomass (MB) can be estimated from gas accumulation data and the amount of substrate truly degraded (Blümmel, Steingass, & Becker, 1997) or from degradation rate and fiber concentration in the feed (Krishnamoorthy & Robinson, 2010).

Although the methanogenic capacity of CT may be related to a direct anti-microbial effect (Morgavi, Forano, Martin, & Newbold, 2010), it may also be due to an indirect effect on ruminal fermentation, generating more efficient fermentation profiles (*i.e.* propionogenic fermentations, Patra & Saxena, 2011).

However, the overall analysis of fermentation profile, digestibility and CH_4p requires further study in order to understand the changes in the metabolic pathway of these fermentation products (Guyader, Ungerfeld, & Beauchemin, 2017). The hypothesis underlying this work is that the CT can be used as a dietary strategy in a forage-based diet to manipulate rumen fermentation to reduce CH_4 production and improve the *in vitro* fermentation efficiency. Therefore, the main objective of this work was to analyze the effects of different CT extracts in digestion, gas production kinetics, and the stoichiometric relation between products derived from *in vitro* fermentation of the forage under study.

Material and methods

Experimental approach

Three sources of CT extracts (*i.e.* Control [without CT], Quebracho [*Schinopsis balansae*] and *Lotus corniculatus*) were incubated with two substrates (*i.e.* ryegrass, *Lolium perenne*, RG and *Megathyrsus maximus*, MM) to evaluate effects of CT on *in vitro* fermentation parameters. Moreover, polyethylene glycol (PEG) was added to incubation (0 and 20 mg per bottle) due to its high affinity for CT, which allowed discriminating the specific action of CT present in the extracts. Briefly explained, sixty bottles (100 mL of capacity) were incubated per run accordingly: three sources of CT × two PEG levels × two substrates × two analytical replicates × two incubation times (24 and 48h), plus 8 blanks (*i.e.* 4 bottles × 2 incubation times) and 4 internal standards. There were three independent incubations (*i.e.* three different runs, statistical replicates, n= 3). The experiment was carried out in the Laboratory of Center for Research and Services in Animal Nutrition (CISNA), School of Agriculture of the University of Buenos Aires (FAUBA).

Substrates and CT extract preparation

Leaves of RG and MM were harvested from the FAUBA experimental field, and fresh ground (*i.e.* once frozen with dry ice with a Foss Tecator 2096 Homogenizer mill; *c.a.* 1 mm particle size) and stored at -20°C until they were used. The DM of each substrate was assessed in an oven to ensure 0.250 ± 0.025 g DM within each bottle. These substrates were characterized (Table 1) by dry matter (DM, 105°C for 4h) and organic matter (OM, 550°C for 5h, Electric furnace, Q.R.L.® Argentina, Association of Official Analytical Chemists [AOAC], 1990, N°930.15), crude protein (CP = total N × 6.25, by Kjeldahl, Pro-Nitro® unit Selecta JP®, Barc., España, N°954.09), ash-free insoluble fiber in neutral detergent, with alpha-amylase ($aNDF_{OM}$, Goering & Van Soest, 1970), ash-free insoluble fiber in acid detergent (ADF_{OM}, N°973.18) and ash-free lignin content (ADL, N°974.17) with an ANKOM 200 Fiber Analyzer Unit (ANKOM® Techn. Corp., Macedon, NY, USA). The differences between $aNDF_{OM}$ and ADF_{OM} and between ADF_{OM} and ADL were used to estimate the hemicellulose and cellulose contents, respectively. The gross energy was determined using a Parr 1241® oxygen bomb calorimeter.

Table 1. Chemical composition (g kg^{-1} DM, except for GE, Mcal kg^{-1}) of the forages used as substrates for *in vitro* fermentation.

Substrate	Ryegrass (*Lolium perenne*)	*Megathyrsus maximus*
ID[1]	RG	MM
DM	159	185
OM	813	856
CP	203	191
$aNDF_{OM}$	338	622
ADF_{OM}	171	322
ADL	9	20
Hem	167	301
Cel	162	301
GE	4.73	4.20

[1]ID, identification; DM, Dry matter; OM, Organic matter; CP, Crude protein; $aNDF_{OM}$, Insoluble fiber in neutral detergent treated with alpha-amylase (ash-free); ADF_{OM}, Insoluble fiber in acid detergent (ash-free), ADL, Lignin treated with sulfuric acid; Hem, Hemicellulose; Cel, Cellulose; GE, Gross energy.

A commercial extract of Quebracho (Unitan Saica®, Argentina) containing 90.5% of CT, was supplied in the form of a fine dry powder. Additionally, Lotus was harvested at vegetative state, after flowering (OM= 911, CP= 211, EE= 21, $aNDF_{OM}$= 487, ADF_{OM}= 314 and ADL= 107 g kg^{-1} DM), dried in an oven with forced air (not exceeding 40°C), ground (1 mm particle size) and washed with distilled water (100 g substrate in 1200 mL of water) in a water bath set at 40°C for 30 min., with 3 minutes agitation and rest

periods. After being filtered with filter paper, the liquid was recovered and stored in a dark glass bottle at 5°C. The CT of both extracts were quantified by the butanol - HCl method (Terril, Rowan, Douglas, & Barry, 1992). The CT contents, measured with the butanol - HCl method, were 2.4 g mL^{-1} for Lotus extract and 0.905 g g^{-1} DM for Quebracho.

In vitro gas production and fermentation products analysis

Substrates (0.250 ± 0.025 g DM) were incubated for 24 or 48h with buffer medium and ruminal liquor (medium: liquor, 9: 1) in a water bath (39°C) in anaerobiosis according to Theodorou, Williams, Dhanoa, Mcallan, & France (1994). Ruminal liquor was obtained from two cannulated adult sheep before the morning feeding (*i.e.* with a standard diet, alfalfa hay: corn grain: 70: 30 plus a mineral supplementation), and later mixed and strained through four layers of cheesecloth into a flask with O_2 free headspace. In addition, 1 mL of CT extract (or distilled water, in Control) at 3% of inclusion (*i.e.* 7.5 mg of CT per 250 mg of substrate incubated) and 1 mL of PEG solution (or distilled water in bottles without PEG, NP) were added to each bottle. Bottle pressure (pressure transducer T443A model, Bailey and Mackey Ltd, U.K.) and gas volume (mL) were measured at 1, 2, 4, 8, 12, 16, 24, 36 and 48 h, in order to characterize the kinetics of accumulated gas production, Lag phase, and instantaneous hourly rates of GP at 1, 10 and 42h of fermentation (mL h^{-1}, T1, T10 and T42, respectively). Non-linear modeling parameters were also studied (Ørskov & McDonald, 1979, $AGP = A + B \times (1 - e^{-c \times t})$). The gas collected was accumulated in vials of 50 mL, for the subsequent analysis of *in vitro* CH_4p reported in g of CH_4 per kg DM incubated and disappeared (CH_4DMi and CH_4DMd) and per kg of disappeared OM (CH_4OMd). CH_4 concentration on fermentation gases was analyzed in an HP 4890 series gas chromatograph (Hewlett Packard® Labs, Palo Alto, CA, USA) equipped with a stainless-steel column (2 m, N Porapak, 80-100 mesh) and an FID detector. Run time was 2 minutes, N gas was used as a carrier (flow rate, 22 mL min^{-1}) and oven, injector and FID temperature were 90, 110 and 250°C, respectively. Moreover, CH_4 concentration was analyzed in blank bottles, to correct CH_4 of experimental bottles arising from the inoculum. Later, CH_4p was estimated according to procedures described by Lopez and Newbold (2007).

Fermentation was interrupted with three drops of saturated thymol and pH was measured (Hanna instruments HI 9025 pH-meter). Aliquots were preserved with sulfuric acid (25%, ratio 1:5, acid: sample) for determination of VFA (Konik chromatograph, model HRGC 5000b, with a FID detector and a Nukol capillary column, 30 mt × 0.32 mm × 0.25 µm layer thickness, Perkin Elmer - Elite FFAP; Part N° 931-635-4, hydrogen as carrier gas [2.4 mL H_2 $min.^{-1}$] and a standard mixture for calibration [Supelco, Cat. N°46975-U]). Another aliquot was preserved with orthophosphoric acid (0.02 N, 1:1, acid: sample) to determine N from ammonia (NH_3-N) concentration (uremia kit, Wiener® lab). Additionally, insoluble residue of each bottle was analyzed with ANKOM® bags (ANKOM® Technology # F57 filter bags), to calculate *in vitro* DM, OM and NDF digestibility (ivDMD, ivOMD and NDFD, Goering & Van Soest, 1970). In addition, the partition factor ($PF = \frac{mg\ ivOMD}{ml\ AGP}$), which reflects the efficiency of synthesis of microbial biomass (ESMB), was calculated (Blümmel et al., 1997). Microbial biomass production ($MB\ production = mg\ ivOMD - (ml\ AGP \times 2.2\ mg/ml)$, mg 100^{-1} mg OM disappeared) was estimated, where 2.2 mg mL^{-1} is a stoichiometric factor in acetogenic substrates (Blümmel, Aiple, Steingas & Becker, 1999).

Experimental design

A randomized complete block design with a factorial 2 × 3 arrangement was used, considering the following model:

$$Y_{ijklm} = \alpha_i + \beta_j + \gamma_k + \delta_l + (\alpha \times \gamma)_{ik} + (\alpha \times \delta)_{il} + (\gamma \times \delta)_{kl} + (\alpha \times \gamma \times \delta)_{ikl} + \varepsilon_{ijklm}$$

where, Y_{ijklm}= variable under study; α_i= substrates (i= 2); β_j= block (run, j= 3); γ_k= CT source (k= 3); δ_l= absence or presence of PEG (l= 2) and ε_{ijklm}= experimental error. Interactions were evaluated and, when confirming that they were not significant, they were eliminated from the model. Three independent runs were carried out (*i.e.* Run≡ Block≡ Statistical replication) separated in time and with a mixed ruminal liquor from two different sheep each time. The variables were analyzed by ANOVA using the GLM procedure (SAS Statistics, Version 9.0. SAS Inst. Inc, Cary, NC, USA). Tukey test (to evaluate differences between substrates and interactions) or Dunnett test (to evaluate the effect of adding CT) with a level of significance $P < 0.05$ were performed. Stoichiometrical relationships were examined by linear regression analysis using the statistical

package GraphPad Prism® 5 (Version 5.01, GraphPad Software, Inc., USA). To account for the incidence of VFA concentration on variables associated with CH_4p and MB an analysis was carried out seeing into experimental factors and VFA composition influence on CH_4OMd, PF and MB.

Results and discussion

Fermentative characteristics

Non-linear models applied to *in vitro* gas production have been used for decades to predict the fermentation kinetics of ruminants' feedstuff, and thus to analyze which proportion is fermented and which is used for microbial growth. In this experiment, the accumulated gas production (AGP, Figure 1) showed that none of the treatments completely achieved the asymptotic phase of gas production in 48 h, and this was particularly noticeable for MM. Parameter A (*i.e.* gas production from the immediately soluble fraction) differed between substrates ($P < 0.05$, Table 2), and RG showed a lower AGP from the insoluble fraction (Parameter B) and a faster rate constant for the insoluble fraction B (Parameter c) than MM. Hence, it is expected that when modeled, Par B had been overestimated especially for MM and consequently resulting in 'B' higher than the one for RG ($P < 0.05$). Moreover, RG presented a lower Lag phase (less than 1 hour, $P < 0.05$), and higher rates in the first hours (T1 and T10). However, after 42h, MM produced more gas ($P < 0.05$). These differences between RG and MM were coherent with the expected quality of these species. On the other hand, both extracts of CT presented higher Par B ($P < 0.05$), even when Lotus extract was added to MM, the lag phase decreased almost 50% compared to the Control (Control= 2.03, Lotus= 1.05, SEM= 0.156, $P < 0.05$, while in RG there were no differences, general mean= 0.97, $P > 0.05$, Table 2). Concomitantly, Lotus increased Par B and Par c, also agreeing with higher instant rates of GP during the first hours (100% at T1, and 27% more at T10), indicating differences among grasses in response to dietary CT inclusion. There was no effect with the addition of PEG ($P > 0.05$).

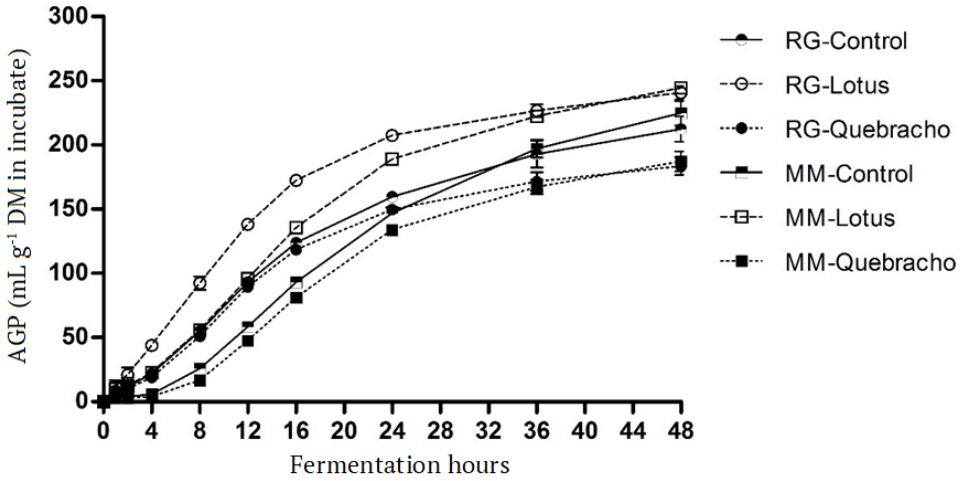

Figure 1. Accumulated gas production (AGP) for *in vitro* fermentation of *Megathyrsus maximus* (MM, squares) and ryegrass (*Lolium perenne*, RG, circles) with extract of condensed tannins of *Lotus corniculatus* (empty symbol, dashed line), Quebracho (*Schinopsis balansae*, dotted line) or without condensed tannins (CT, Control, solid line) after 48 h of incubation. Analysis of non-linear modeling parameters according Ørskov and McDonald (1979), $AGP = A + B \times (1 - e^{-c \times t})$ for the interaction Substrate × CT: Par A (mL, $P > 0.05$, SEM= 1.32), Par B (mL, $P > 0.05$, SEM= 7.7) and Par c (h^{-1}, $P < 0.001$, SEM= 0.012).

Higher CH4p of MM with respect to RG ($p < 0.05$, Table 2) was in agreement with the premise that C4 grasses produce more CH4 than C3 grasses (Archimède et al., 2011). It has been shown that depending mainly on the dose and type, the addition of CT can reduce CH4p (Knapp, Laur, Vadas, Weiss, & Tricarico, 2014) as shown in previous *in vitro* studies (Supapong et al., 2017) as well as in *in vivo* experiments (Piñeiro-Vázquez et al., 2018). This mitigating effect of the CT may be due to the direct effect on the ruminal fermentation profiles that these compounds have, possibly associated with a reduction in the protozoan (Mohammadabadi & Jolazadeh, 2017) or methanogenic population (Puchala et al., 2012; Tavendale et al., 2005).

One of the mechanisms of action of CT in these populations may be linked to inhibitions of morphological changes of the cell wall (Jones, McAllister, Muir, & Cheng, 1994). Contrary to what was expected, in the present study both sources of CT increased CH4p compared to Control ($P < 0.05$, Table 2); however, no effect was observed

with the addition of PEG ($P > 0.05$). These differences could arise in part, from dissimilar fermentation profiles, which would be defined stoichiometrically by the composition of the fermentation products (Blümmel et al., 1999), *i.e.* a higher concentration of VFA profiles. Lotus generated a higher concentration of acetic (Ac), propionic (Prop), butyric (But) acids, and total VFA (tVFA, 24 and 48 h, $P < 0.05$). On the other hand, the inclusion of Quebracho did not generate differences ($P > 0.05$) vs. Control at 24 h (Figure 2a and b). However, at 48 h, an increase in Ac led to greater tVFA (Figure 2c). Consequently, the Ac: Prop ratios of Lotus decreased while in Quebracho they increased (Figure 2d). These acetogenic profiles found with Quebracho treatment would release more H+ with methanogenic potential to the medium (Janssen, 2010).

Table 2. Methane production (g kg^{-1}) after 24 or 48h of fermentation, affected by DM incubated (CH_4-DMi) or digested (CH_4-DMd) and OM digested (CH_4-OMd), net accumulated gas production (NAGP), with the parameters of a non-linear model (Par A, B and c, Ørskov & McDonald, 1979), Lag phase (h) and hourly GP rates at 1, 10 and 42 h (mL h^{-1}, T1, T10 and T42) of two forages with the addition of extract of condensed tannins (CT).

	24h of incubation			48h of incubation			NAGP= A + B × (1 - $e^{-c \times t}$)			Lag phase	Hourly GP rates		
	CH_4-DMi	CH_4-DMd	CH_4-OMd	CH_4-DMi	CH_4-DMd	CH_4-OMd	Par A (mL)	Par B (mL)	Par c (h^{-1})		T1	T10	T42
Substrates[1] (n=18)													
MM	12.5	16.1	16.3	14.7	17.4	17.2	-19.7	275	0.04	1.58	6.2	8.7	1.9
RG	11.1	13.1	12.2	11.8	13.2	12.5	-16.4	243	0.10	0.97	16.5	10.0	1.3
SEM	0.63	0.78	0.83	0.68	0.82	0.83	1.08	6.3	0.009	0.079	1.53	0.33	0.06
Condensed tannins source[2] (n=12)													
Control	9.5^a	11.7^a	11.5^a	10.7^a	12.3^a	12.0^a	-17.7	231^a	0.05^a	1.55^a	6.5^a	8.6^a	1.3^a
Lotus	14.0^b	17.2^b	16.4^b	13.9^b	16.0^b	15.6^b	-16.6	278^b	0.10^b	0.90^b	19.5^b	11.0^b	1.5^b
Quebracho	11.8^b	15.0^b	14.8^b	15.1^b	17.7^b	17.0^b	-20.0	267^b	0.06^a	1.38^a	8.0^a	8.4^a	2.0^b
SEM	0.77	0.96	1.01	0.84	1.01	1.03	1.32	7.7	0.012	0.137	1.87	0.40	0.07
Significance[3]													
Substrate	*	***	***	***	***	***	**	***	***	***	***	***	***
CT source	***	***	***	***	***	***	δ	***	***	***	***	***	***
PEG	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Subst × CT	ns	ns	ns	ns	ns	ns	ns	ns	***	**	ns	ns	ns

[1]MM, *Megathyrsus maximus;* RG, Ryegrass (*Lolium perenne*); SEM, Standard error of the mean; [2]Control, without CT; Lotus, *Lotus corniculatus* extract, Quebracho, *Schinopsis balansae* commercial extract. [3]Subst, substrate; PEG, polyethylene glycol addition; ns, not significant ($p > 0.05$); δ, $p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Comparisons according to Tukey for substrates, PEG and interactions ($P < 0.05$) and according to Dunnett for the CT Source. Different letters in each column indicate differences to Control ($P < 0.05$).

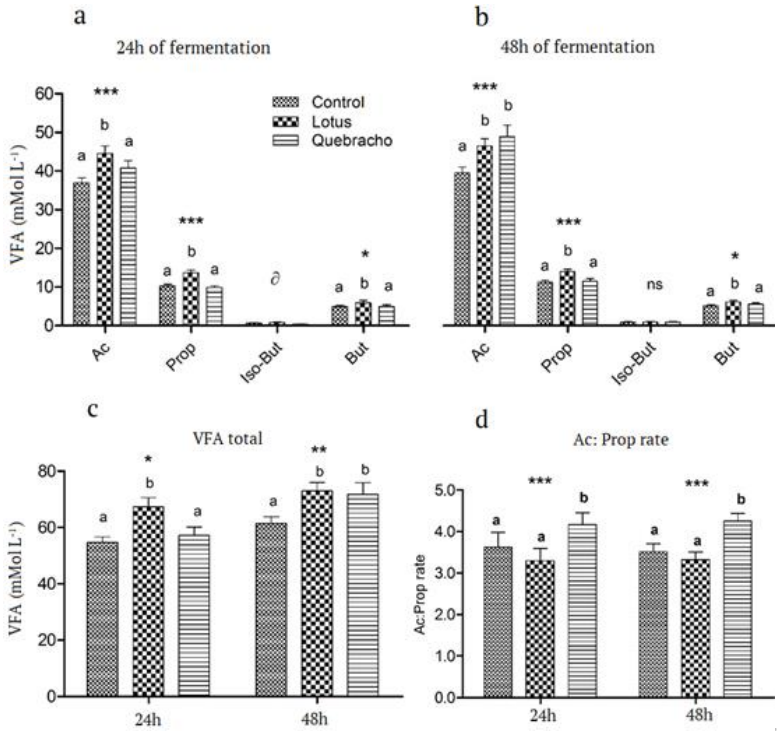

Figure 2. Profiles of volatile fatty acids production (VFA; acetic, propionic, butyric and iso-butyric; Ac, Prop, But and Iso-But, respectively; figures above), total VFA, and acetic: propionic rate (Ac: Prop rate; figures below) with extract of condensed tannins (CT) of *Lotus corniculatus*, Quebracho (*Schinopsis balansae*) or without CT (Control) after 24 and 48h of incubation. ns, not significant ($P > 0.05$); δ, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Comparisons according to Dunnett. Different letters in same group of columns indicate differences to Control ($P < 0.05$). SEM= 1.66, 0.67, 0.16, 0.34 (Figure a) and SEM= 2.18, 0.57, 0.14, 0.32 (Figure b) for Ac, Prop, Iso-but, and But, respectively. SEM= 2.51, 2.98 (Figure c) and SEM= 0.13, 0.08 (Figure d), for 24 and 48h of fermentation, respectively.

Moreover, the analysis of VFA showed differences for substrates only after 48 h of fermentation for all the VFA (RG > MM, $P < 0.05$, Figure 3), but without modifications in the Ac: Prop ratio (general mean= 3.7, $P > 0.05$). PEG did not produce any effect (24 and 48 h, $P < 0.05$, data not shown).

Condensed tannins act as inhibitors of protein and carbohydrate degradation (Patra & Saxena, 2011) and possess great potential to mitigate fermentation gases (Knapp et al., 2014). The antimicrobial effect observed in *in vitro* works seems to be associated with the changes in fermentation produced by the inclusion of CT (Mohammadabadi & Jolazadeh, 2017; Puchala et al., 2012). Numerous studies have focused on the depression of ruminal fiber digestion associated with the inclusion of CT as a limitation in their use (Patra & Saxena, 2010), although this may be an indirect cause of the mitigating potential of GHGs by these compounds. In this work, a decrease in NDFD, ivDMD, and ivOMD caused by the addition of Quebracho was observed at 24 h ($P < 0.05$, Table 3), while the addition of PEG increased digestibility parameters ($P < 0.05$) indicating an effect of CT (Baba, Castro, & Orskov, 2002). This fact was also shown in other works of this research group (Cantet, Neumann Reiter, Colombatto, Wawrzkiewicz, & Jaurena, 2018).

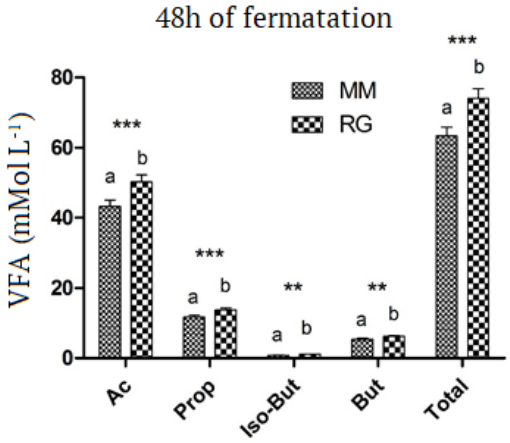

Figure 3. Concentration of volatile fatty acids production (acetic, propionic, butyric, iso-butyric, and total VFA; Ac, Prop, But, Iso-But and Total, respectively) from *in vitro* fermentation of *Megathyrsus maximus* (MM) and ryegrass (*Lolium perenne*, RG) after 48 h of incubation. Comparisons according to Tukey. Different letters in the same group of columns indicate differences ($P < 0.05$). **, $P < 0.01$; ***, $P < 0.001$. SEM= 1.78, 0.47, 0.11, 0.26, and 2.43 for Ac, Prop, Iso-but, But, and Total respectively.

Table 3. In vitro dry matter digestibility (ivDMD), organic matter digestibility (ivOMD, g kg^{-1} DM), NDF digestibility (NDFD, g kg^{-1} NDF), and digestible NDF (dNDF, g NDF kg^{-1} DM) after 24 and 48h of fermentation of two forages with condensed tannins extract (CT) of *Lotus corniculatus*, *Quebracho sp.* or without CT (Control), with presence or absence of polyethylene glycol (PEG or NP).

	24h of incubation				48h of incubation			
	ivDMD	ivOMD	NDFD	dNDF	ivDMD	ivOMD	NDFD	dNDF
				Substrates[1] (n=18)				
MM	776	769	672	122	843	850	799	145
RG	852	893	800	119	896	942	908	135
SEM	5.7	6.5	13.6	2.4	3.9	4.3	7.9	1.6
				Condensed tannins source[2] (n=12)				
Control	824[a]	839[a]	750[a]	123[a]	873	900	859	141
Lotus	824[a]	845[a]	757[a]	124[a]	870	897	856	140
Quebracho	794[b]	810[b]	701[b]	115[b]	866	891	844	139
SEM	7.0	8.0	16.7	2.9	4.7	5.2	9.7	1.9
				PEG addition (n=18)				
NP	807	822	722	118	868	895	851	140
PEG	820	840	750	123	871	897	855	140
SEM	5.7	6.5	13.6	2.4	3.9	4.3	7.9	1.5
				Significance[3]				
Substrate	***	***	***	ns	***	***	***	***
CT source	***	***	**	**	ns	ns	ns	ns
PEG	*	*	*	δ	ns	ns	ns	ns

[1]MM, *Megathyrsus maximus;* RG, Ryegrass (*Lolium perenne*); SEM, Standard error of the mean; [2]Control, without CT; Lotus, *Lotus corniculatus* extract, Quebracho, *Schinopsis balansae* commercial extract. [3]ns, not significant ($P > 0.05$); δ, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Comparisons according to Tukey for substrates, PEG and interactions ($P < 0.05$) and according to Dunnett for the CT Source. Different letters in each column indicate results different to Control ($P < 0.05$). There were no interactions ($P > 0.05$), whereby they are not shown in the table.

It was observed that the addition of Quebracho extract reduced the NDFD (after 24 and 48h of fermentation) but increased CH_4p. The response detected after the addition of PEG suggests that, although the reduction of the NDFD was due to the presence of CT, this was not related to higher CH_4p. One possible explanation would be that in the extracts used, there were compounds that were different from CT which could alter the methanogenic potential of different forages, without altering their capacity to inhibit fiber degradability. Yet, a more likely explanation might reside in differences in tannin molecular weight and/or monomeric composition (Barahona Rosales et al., 2003) that lead to highly contrasting nutritional impacts among CT from different sources. However, depressing effects of CT on CH_4p and NDFD *in vitro* were found by Jayanegara, Makkar, and Becker (2015) with extracts of Quebracho (*Schinopsis balansae*) and mimosa (*Mimosa tenuiflora*). These results show that there still exists a considerable uncertainty about the effectiveness of different sources of CT to reduce fermentative CH_4p and there is evidence that the potential beneficial effect on ruminal fermentation mostly depends on the source of CT, the concentration (Carreño, Hervás, Toral, Belenguer, & Frutos, 2015), the type of solvent used and extraction procedure (Barahona Rosales et al., 2003) and the tannin characteristics (Huang et al., 2010).

Energy distribution among the final fermentation products

Analysis of products generated during *in vitro* fermentation provides valuable information of the ruminant feed under evaluation (Guyader et al., 2017). The stoichiometry developed by Wolin (1960) specifies that both the GP and its composition (*i.e.* CO_2 and CH_4) can be calculated from the proportions of Ac, Prop, and But obtained *in vitro* (Blümmel et al., 1999). While the production of Prop from pyruvate consumes hydrogen, the production of Ac and But releases CO_2 and free H^+ to the ruminal environment that escapes from the rumen mainly as CH_4 (Janssen, 2010). This fact was confirmed by our results after 24 h of incubation as CH_4-OMd decreased in association with Prop (-0.75 mL CH_4 g^{-1} OMd, $P = 0.31$) and increased in concordance with Ac (0.22, $P < 0.001$) and But (0.77 mL CH_4 g^{-1} OMd, SE= 0.729, $P < 0.001$) concentrations. Moreover, the comparison between the stoichiometrically calculated GP according to Blümmel et al. (1999) and measured, pressure-uncorrected gas volumes at 24h of total data were significant (slope 95% confidence interval of 0.065 to 0.244; $P < 0.05$), though the r^2 coefficient accounted for just a 15% of total variation (Sy.x = 8,99, Figure 4a). This relation was greatly influenced by the MM AGP estimate (Figure 4b).

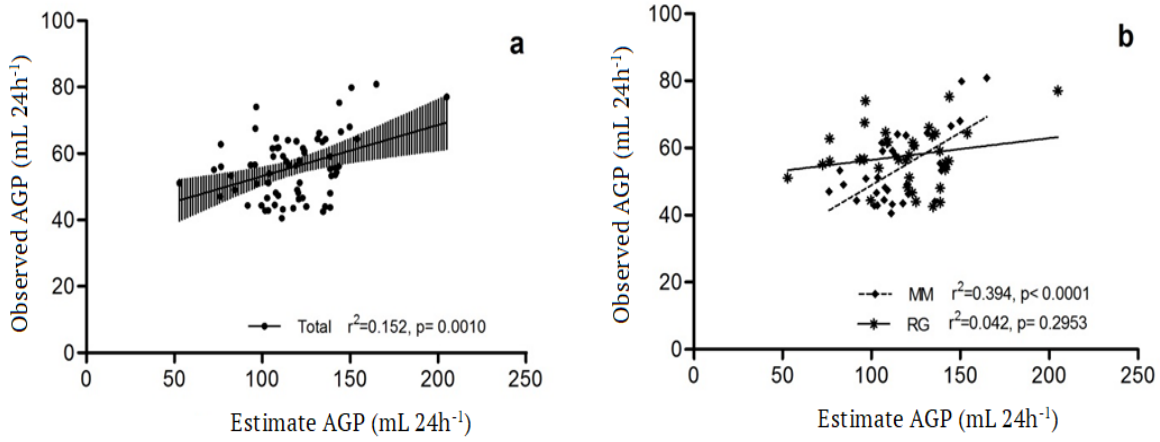

Figure 4. Relationship between stoichiometrically calculated accumulated gas production (AGP) and measured pressure-uncorrected gas volumes of total data (circles, bars in the line indicate 95% confidence interval of the slope, a), *Megathyrsus maximus* (MM, asterisks, linear regression with dashed line) and ryegrass (*Lolium perenne*, RG, diamonds, linear regression with solid line, b) after 24h of incubation.

As it was expected, the inclusion of VFA into statistical models of the analysis showed that they influenced CH_4p per unit of fermented organic matter (*i.e.* OMd) by run, substrate, and CT source ($P < 0.01$; Table 4) and by Ac ($P < 0.001$) and But ($P < 0.001$) concentrations, as a probable consequence of the availability of hydrogen produced during OM fermentation. The CH_4-OMd was raised in concordance with Ac (0.22 mL g^{-1} OMd, SE= 0.173, $P = 0.22$) and But concentrations (0.77 mL g^{-1} OMd, SE= 0.729, $P = 0.30$). In addition, it was found that PF and MB were affected by CT source and VFA composition, though for MB a triple interaction (Substrate × CT × PEG) was detected (RG and Lotus, N= 33, P= 43, $P = 0.005$; RG and Quebracho, N= 51, P= 43, $P = 0.005$, Table 4). However, it could be seen that beyond the direct effect of VFA there were residual effects associated with substrates and CT sources.

Table 4. Methane production (CH_4-OMd, g kg^{-1} digested organic matter), partition factor (PF), microbial biomass (MB) produced after 24 or 48 h of fermentation, accounted for acetic, propionic, and butyric acids concentrations as regressors and runs (independent incubations), substrate, condensed tannin source (CT) and polyethylene glycol (PEG) as classificatory factors.

	24h of incubation			48h of incubation		
	CH_4-OMd	PF	MB	CH_4-OMd	PF	MB
			Substrates[1] (n= 18)			
MM	16	4.4	47	17	3.4	34
RG	12	4.3	47	12	3.7	39
SEM	0.5	0.09	0.6	0.6	0.06	1.1
			Condensed tannins source[2] (n= 12)			
Control	12^{a}	5.0^{a}	54^{a}	12^{a}	4.1^{a}	45^{a}
Lotus	16^{b}	3.5^{b}	37^{b}	16^{b}	3.2^{b}	30^{b}
Quebracho	14^{ab}	4.6^{a}	49^{b}	16^{b}	3.4^{b}	35^{b}
SEM	1.6	0.14	1.0	1.0	0.22	1.9
			Significance[3]			
Acetic acid	***	**	***	*	***	***
Propionic acid	ns	***	***	ns	ns	*
Butyric acid	***	ns	*	***	**	*
Run	***	**	***	***	δ	ns
Substrate	***	ns	ns	***	**	***
CT source	**	***	***	*	***	***
PEG	ns	ns	ns	ns	ns	ns
CT × PEG	ns	ns	**	ns	ns	ns
Subst × CT	ns	ns	δ	ns	ns	ns
Subst × CT × PEG	ns	ns	*[4]	ns	ns	ns

[1]MM, *Megathyrsus maximus;* RG, Ryegrass (*Lolium perenne*); SEM, Standard error of the mean; [2]Control, without CT; Lotus, *Lotus corniculatus* extract, Quebracho, *Schinopsis balansae* commercial extract. [3]ns, not significant ($P > 0.05$); δ, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Comparisons according to Dunnet. [4]For RG and Lotus, N= 33, P= 43, $P = 0.005$; for RG and Quebracho, N=51, P= 43, $P = 0.005$.

In order to improve the fermentative characteristics of forages, additives are expected to reduce CH_4p and promote a lower Ac: Prop ratio, which may improve ESMB (Goel, Makkar, & Becker, 2008). This could be valid, although partially since the reduction of CH_4p is often associated with a decrease in the degradability of OM in general and of fiber in particular (Jayanegara et al., 2015). Contrary to expectations, the Quebracho extract reduced the NDFD, without changes in the GP but it increased the CH_4p, although this greater methanogenic potential could be explained stoichiometrically by the increase in the Ac: Prop ratio (Blümmel et al., 1999). Conversely, the increase in CH_4p in Lotus was not accompanied by changes in digestibility. Although this extract induced an increase in the tVFA compared with the Control (Figure 2c), this increment was proportionally higher in Prop (33%) than in Ac (21%), generating an Ac: Prop ratio 8% lower at 24h (Figure 2d), analogous to 48 h. Even though we cannot explain stoichiometrically the highest CH_4p with these VFA profiles, it can be inferred that the highest CH_4p of Lotus was associated with a higher GP with the same digestibility, compared with the Control. Even though there are references of the improvement of ESMB reflected in high PF (Baba et al., 2002) by the addition of CT, both extracts utilized here (*i.e.* Quebracho and Lotus) reduced PF and the estimate MB ($P < 0.05$, Table 4). Moreover, this is not always related to a lower concentration of CH_4 on the fermentation gases, but rather to a lower GP (Bueno et al., 2015).

Additionally, the higher MB productions associated with the addition of CT suggest that the presence of these compounds in the rumen would produce an increase in the flow of microbial N to the duodenum (Nguyen, Wanapat, Phesatcha, & Kang, 2017), increasing the efficiency of recycled urea for the rumen due to a reduction on protein degradation, with a lower concentration of ruminal NH3-N (Nguyen et al., 2017). In this work, RG presented higher values of NH3-N (7.9 and 6.4 at 24 h and 8.9 and 11.7 at 48 h for RG and MM, SEM= 0.60, $P < 0.05$) and the highest concentration of NH3-N found after 48 h of fermentation in treatments with CT (9.2, 10.9 and 10.8, for Control, Lotus and Quebracho respectively, SEM= 0.73, $P < 0.05$) is consistent with the low MB production and suggests a lack of effect of the CT, at least partially, in the protection of protein against microbial attack.

Conclusion

Although CT of Quebracho had a negative effect on fiber digestibility, contrary to expectations, these treatments produced more CH_4. The higher CH_4p in the treatments containing extracts of Quebracho and

Lotus cannot be attributed to CT presence, due to the lack of effect of PEG, suggesting that other soluble compounds present in the extracts promoted this increase of CH_4p. The stoichiometric analysis indicated that, although there was no evident relationship between the VFA and CH_4p, the higher CH_4p caused by Quebracho extract would be associated with VFA profile (*i.e.* more acetogenic than the Control), while in Lotus they are related to the higher production of tVFA and higher GP. Finally, the lower values of PF with higher concentrations of NH_3-N in the CT treatments evidence a lack of protection of these compounds in the protein ruminal degradation of the forages under study.

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