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Effects of Condensed Tannins from Quebracho Extract on the Kinetic of *in vitro* Gas Production on *Trifolium repens*, *Lotus corniculatus* and *Lolium perenne*

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Abstract: The aim of this work was to study the effect of condensed tannins (CT) on the kinetic of gas production *in vitro*, on the three main species of Azorean pasture: *Trifolium repens*, *Lotus corniculatus* and *Lolium perenne*, using Quebracho extract as the source of CT, and also to calculate total tannins and condensed tannins on the species studied. *Lotus corniculatus*, *Lolium perenne* and *Trifolium repens* were tested *in vitro* with the inclusion of Quebracho extract in doses of 0%, 2.5% and 5% DM. The total content in tannins was determined in the 3 species. We have found, in comparison with standard tannic acid, that tannins exist in the flower of *T. repens* (0.81 equivalents of tannic acid) and in *L .corniculatus* (1.07 equivalents of tannic acid) but not in *L .perenne* (0 equivalents of tannic acid). The concentration of condensed tannins in the 3 species was: 0.34 mg/mL for *T. repens*; 0.83 mg/mL for *L. corniculatus* and 0 mg/ml for *L. perenne*. We verified that a significant reduction ($P < 0.05$) of gas production happened for doses of 5% of CT in comparison with the other two concentrations: 0% and 2.5% of CT. The results of this study express a reduction of the fermentation rate, which implies a reduction of gas production, so, a reduction of methane emission to the atmosphere and an increase of exploitation of the protein by ruminants.

Key words: Quebracho, condensed tannins; *in vitro* gas production, *Trifolium repens*, *Lotus corniculatus*, *Lolium perenne*.

1. Introduction

With the growing concern about global warming and global climate changes, on which the cattle farm has an important role because of the production of greenhouse-gases, like methane (CH_4), it is very important to find ways to reduce the production of such gases, including the use of additives such as condensed tannins from Quebracho.

Condensed tannins are well recognized for their ability to: (a) complex with soluble rumen proteins, (b) reduce the degradation of protein to ammonia in the rumen and (c) allow more dietary protein to flow to the small intestine [1, 2] increasing the supply of digestible protein to the host [3, 4].

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Dietary CT precipitate proteins in the rumen prevent bloat and reduce protein degradation. CT bind to plant proteins when released from plant cells, lowering their solubility and ammonia concentrations in rumen fluid [5, 6] and increasing the quantity of non-ammonia N and amino acids reaching the small intestine [7].

Because of the particular characteristics of CT and despite other studies on this field, the aims of this work were to study the effect of CT from Quebracho extract on the kinetic of *in vitro* gas production and to determine the quantity of total tannins and CT on the three main species of Azorean pasture, which was never studied before.

2 Materials and Methods

Pasture (*T. repens*, *L. corniculatus*, *L. perenne*) samples were sampled on days 16, 19 and 22 of each period of the assay (22 days each), in order to determine their chemical parameters, total tannins and condensed tannins and to study the kinetic of the *in vitro* gas production, according to the method described by Menke [8].

2.1 Chemical Composition of Trifolium repens, Lotus corniculatus and Lolium perenne

Pasture samples were collected, preserved and assayed to determine chemical parameters as the previously described [9].

Fibre analyses of each sample were completed as described by Van Soest et al. [10]. Both neutral detergent fibre (NDF) and acid detergent fibre (ADF) are expressed on an ash-free basis. Lignin was determined using the sulphuric acid procedure proposed by Van Soest et al. [10]. Crude protein (CP) was determined with AOAC [9] procedures.

2.2 Total Tannins and Condensed Tannins

The total content of tannins studied on the three species was determined by the radial diffusion assay, according to Hagerman [11]. In this method, tannin molecules migrate through agarose gel which is impregnated with the protein, bovine serum albumin (BSA). The tannin-protein complex is formed in the gel which appears as a ring. The diameter of the ring is a measure of protein precipitation/binding capacity of tannins [11].

A calibration curve was prepared using tannic acid (Merck GmbH, Darmstadt, Germany).

Total phenols were calculated as tannic acid equivalents (d^2).

For the determination of CT (proanthocyanidins), authors used the acid-butanol method. It is based on oxidative depolymerization of condensed tannins in butanol-HCl reagent [12] for the same species, using as a standard purified Quebracho. The purification of crude quebracho tannin yield to a condensed tannin (5-deoxyproanthocyanidin)—enriched fraction. This method was based on that of Asquith and Butler [13].

2.3 In vitro Gas Production

In vitro gas production (GP) technique simulates the rumen fermentation process and it has been used to evaluate the potential of feeds to supply nutrients to ruminants. It is similar to the ruminal process as gas (CO_2 and CH_4) is produced from the carbohydrate fermentation.

The *in vitro* GP assay was used to determine the kinetic of gas from the three species studied, with and without CT from Quebracho extract. Authors used 0%, 2.5% and 5% DM of Quebracho extract to study the effect of this compound on the ruminal microorganisms and the consequent gas production.

For the assays, the three species were used in duplicate with sheep rumen liquor as inoculum. Each assay was repeated three times (runs). Blanks were used for each inoculum to measure the fraction of total gas production due to substrate in inocula, and these values were subtracted from the total to obtain net GP. All treatments, for each assay, were incubated simultaneously in all runs [14].

Rumen digesta samples were obtained from two sheep and pooled together in order to achieve homogenous inocula. Rumen digesta was collected at 08:00 (before the morning feeding), placed in a container that was sealed immediately, and transported to the adjoining laboratory. The preparation of buffer solutions and rumen inocula was as described by Menke and Steingass [15]. It is recommended to take the rumen fluid before feeding, because it is most constant in its composition and activity. It is also advisable to take the rumen fluid mixture from at least two donor animals as this guarantee a greater constancy of activity [8, 15].

The initial gas volume was recorded after 4, 8, 12, 24, 48, 60, 72, 84 and 96 hours of incubation.

This gas production represents the kinetic of the rumen apparent GP and is expressed by the McDonald [16] equation.

2.4 Calculations and Statistical Analysis

Gas production profiles were obtained after fitting the data to the exponential equation of McDonald [16]:

$$p = a + b\ (1 - exp^{-c\ t}) \quad (1)$$

Where: p represents the gas production at time t; $a + b$ the potential gas production, and c the rate constant.

In vitro gas production from the three species studied with different CT was subjected to analysis of variance (ANOVA) using SPSS 15.0 for Windows.

3 Results and Discussion

3.1 Chemical Composition of Trifolium repens, Lotus corniculatus and Lolium perenne

The chemical composition of *Trifolium repens, Lotus corniculatus and Lolium perenne* is presented in Table 1.

Lolium perenne has a greater content of fibre and a lower content of protein when compared with *Trifolium repens* and *Lotus corniculatus*. Those differences are common for the three species studied (grass—*L. perenne* and legumes—*T. repens* and *L. corniculatus*).

Compared to the grass, legumes were higher in CP content and poorer in fibre content.

3.2 Total Tannins and Condensed Tannins (CT)

The radial diffusion assay is a biological assay that measures the ability of tannin to precipitate bovine serum albumin (BSA). The ability to precipitate BSA is related to the molecular weight, hydroxylation pattern, interflavan binding, and other structural characteristics of tannin. According to a study by Nelson et al. [17], the total phenolics assay was the best predictor of tannin antimicrobial activity. High levels of total phenolics in the purified tannin extracts were strongly correlated with antimicrobial activity. In this study (Table 2) we found tannins both on *Trifolium repens* flowers and *Lotus corniculatus*. The levels of total phenolics are similar to a study by Hedqvist et al. [18] where tannin concentrations of the different varieties ranged between 0.3 ± 1.0%.

Once we detected tannins on two (*Trifolium repens* flowers and *Lotus corniculatus*) of the three species, we used the acid-butanol assay to determine the concentration of CT, according to the method of Porter et al. [12], using purified Quebracho as a standard.

The content of total CT was higher in *Lotus corniculatus* than in *Trifolium repens* flowers (Table 3).

3.3 In vitro Gas Production

Figs. 1-3 present the results of *in vitro* gas production for the three different species studied, with three different CT from Quebracho extract concentrations (0%, 2.5% and 5%).

The gas production was significantly ($P < 0.05$) affected by the addition of CT from Quebracho extract, when compared with the control sample (0% of CT from Quebracho).

Tannins were shown to bind proteins in the rumen environment and offer considerable potential for reducing ruminal degradation of protein and increasing protein digestion in lower gut [7]. Accordingly, tannins might increase the efficiency of N utilization [7, 19]. The CT from Quebracho extract causes a depressing effect on microbial activity, consequently in the *in vitro* gas production [20] and in the efficient growth of such microorganisms [21].

Table 1 Chemical composition (% DM) of the species studied.

Species	Components			
	CP	NDF	ADF	Ash
Lolium perenne	13	64.29	35.48	5.78
Trifolium repens	19.94	37.01	32.84	7.85
Lotus corniculatus	17.5	41.44	34.79	7.13

Table 2 Tannic acid equivalents (% DM) determined with the radial diffusion assay by Hagerman [11].

Species	Tannic acid equivalents
Lolium perenne	0
Trifolium repens	0.81
Lotus corniculatus	1.07

Table 3 Condensed tannins (mg/mL) of the species studied.

Species	Condensed tannins
Lolium perenne	0
Trifolium repens	2.73
Lotus corniculatus	5.51

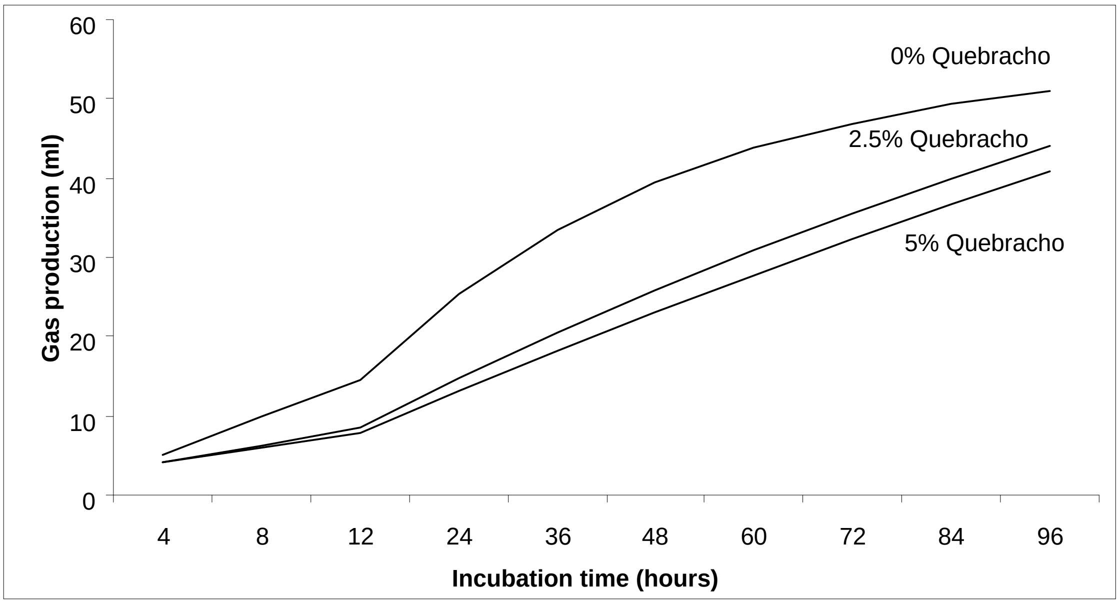

Fig. 1 Graph of accumulated gas production (mL) over a 96 h period for *L. perenne*.

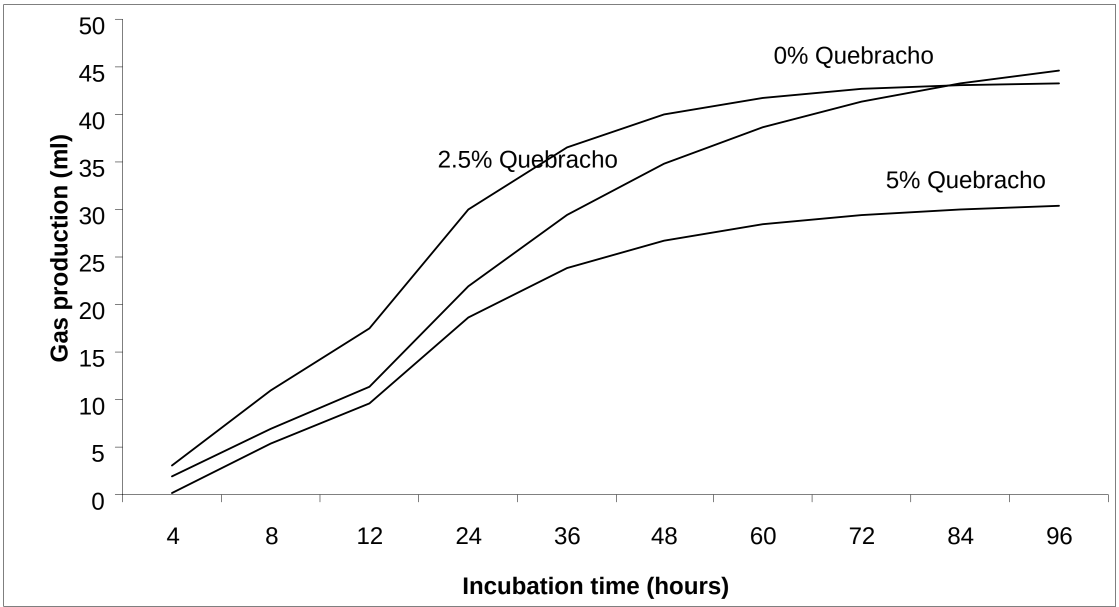

Fig. 2 Graph of accumulated gas production (mL) over a 96 h period for *T. repens*.

In this study (Figs. 1-3) we verified that a significant reduction ($P < 0.05$) of gas production happened for doses of 5% of CT in comparison with 0% of CT. We also find that there is a difference between the total gases produced by the different species studied and that the gas production is lower on species with more CT, such as *L. corniculatus.*

The non-significant differences between the other doses (0% and 2.5%) are consistent with studies by Beauchemin et al. [22], who also noticed that about 2% of CT in the diet was not sufficient to cause any effect on the *in vitro* gas production. Beauchemin et al. concluded in their study that feeding up to 2% of the dietary DM as Quebracho tannin extract failed to reduce enteric methane emissions from growing cattle, although the protein-binding effect of the condensed tannins was evident. They also found no significant differences between 0% and 2% of CT from Quebracho extract.

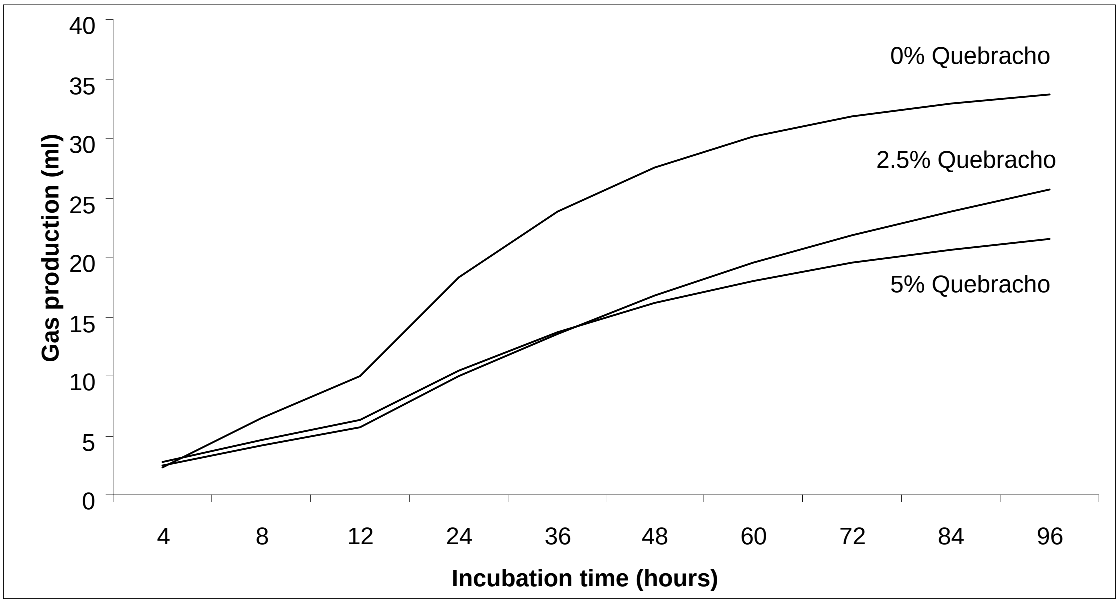

Fig. 3 Graph of accumulated gas production (mL) over a 96 h period for *L. corniculatus*.

Protein in white clover (*Trifolium repens*) is poorly used by ruminants because of its extensive degradation to ammonia in the rumen. However, white clover produces CT in its flowers, which can reduce rumen proteolysis [23]. According to studies, there is a greater amount of protein that reaches the small intestine when animals are fed with white clover, compared to those fed with grass.

In this study, *T. repens* (Fig. 2) produced more gas than *L. corniculatus* (Fig. 3), as expected and less than *L. perenne*, probably because of the higher proportion of flowers than leaves. There is a clear effect of CT that shows a reduction on gas production ($P < 0.05$) between 0% and 5% of CT from Quebracho extract, but, as proposed by a study by Burggraaf et al. [24], the effects of increasing proportions of clover DM as flowers (and therefore floral CT) on soluble protein, ammonia and volatile fatty acid(VFA) concentrations *in vitro*, may have a minimal impact and increasing flowering was not likely to substantially improve the nutritive value of white clover fed to dairy cows especially because of the limitation of occurrence of the adequate concentration of clover flower on the pasture [24]. It is very likely that the same can be concluded for this study once the gas produced with 5% of CT on *T. repens*, comparing to *L. corniculatus* with 0% is not significant.

The yellow trefoil (*L. corniculatus*) was the specie that produced less gas (Fig. 3) and had the higher CT content. As reported by Min et al. [25]. *L. corniculatus* CT reduced the bacterial proteolysis and reduced the growth of proteolytic rumen bacteria.

According to Barry and McNabb [1], *in vitro* experiments have shown that the action of CT in *Lotus corniculatus* slows down the rates of both solubilisation and degradation of forage proteins by rumen microorganisms, especially that of the principal leaf protein, ribulose-bisphosphate carboxylase/oxygenase. In trials where *L. corniculatus* was fed as a sole diet, the CT reduced rumen ammonia concentrations and N digestibility by 7 ± 8% [7, 26]. These results are consistent with our results, since we found significant differences in the reduction of gas production at a percentage of 5% of CT.

The *L. perenne* (Fig. 1) specie produced more gas than the other two species which can be explained by the absence of tannins. But in this case there is also a significant difference between the 0% CT curve and the 5% CT curve and this is a good way to see the effects of CT on the production of gas and on the rumen flora.

4. Conclusions

The results of the *in vitro* assay demonstrated that supplementing the diet with 5% of CT may reduce gas production in the rumen, decreasing the occurrence of bloat and increasing the amount of protein *by-pass* into the small intestine, improving animal performance and decreasing the amount of gases released in the atmosphere.

The use of forages mixed with Quebracho extract in diets containing excess of nitrogen may be beneficial due to the capacity of CT to bind with proteins, which reduce the digestion of nitrogen.

By choosing to use the pasture as a supplement of *by-pass* protein, it is preferable to choose species that have high CT content, such as *L. corniculatus* or *T. repens* at the time of flowering. However, the practical value of high proportions of *T. repens* in pastures is limited by the incidence of bloat, and also because of excessive protein degradation to ammonia, resulting in large ruminal losses and inefficient utilisation of dietary protein [27]. This is a delicate balance, especially when it comes to animals that graze all year. It is therefore necessary to resort to further studies to help determine the proper proportion of species in the diet in order to maximize the potential of CT and minimize the adverse effects of excessive degradation of protein of *T. repens*.

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