Ruminal ammonia concentration and fermentation kinetics of commercial herbal feed additives with amino acids

Cinética de liberación de nitrógeno amoniacal y fermentación ruminal de aditivos para piensos a base de hierbas con aminoácidos

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Abstract

The objective of this study was to characterize the chemical composition of rumen fermentation while estimating it's *in vitro* protein degradation (from ruminal ammonia concentration) and kinetics regarding two herbal feed plant additives. The tested herbal mixtures were elaborated with *Phaseolus mango* and *Linum usitatissimum,* providing lysine (Lys) and *Trigonella foenum-graecum* and *Allium sativa,* providing Methionine (Met). They were compared to alfalfa (*Medicago sativa*) and solvent extracted soybean meal (*Glicine max*), as standard sources of protein using the *in vitro* gas production technique modified to estimate N-NH_3, recording fermentation kinetics and dry matter digestibility (72 h), in a completely randomized design followed by Tukey test. Ruminal ammonia concentration in the herbal mixtures was lower (P<0.05) than in the standard protein sources, indicating that protein from herbal mixtures could resist ruminal degradation. Herbal additives with Lys or Met showed minimum N-NH_3 concentration in the first 4 h of incubation. At 8 h, the concentration was 0.27 and 0.54 mg dL^{-1} for the herbal products with Lys and Met, significantly lower than solvent extracted soybean meal and alfalfa (1.15 and 2.24 mg dL^{-1} respectively, P<0.05).

Keywords

Allium sativa • Feed plant additive • *Linum usitatissimum* • lysine • methionine • ammonia nitrogen • *Phaseolus mango* • protein • rumen • *Trigonella foenum-graecum*

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Resumen

El objetivo del estudio fue caracterizar la composición química, estimar la degradación de la proteína *in vitro* (a partir de la concentración de N amoniacal) y los parámetros de cinética de fermentación ruminal de dos aditivos herbales. Las mezclas herbales probadas están elaboradas con *Phaseolus mango* y *Linum usitatissimum*, para aportar lisina (Lis), y con *Trigonella foenum-graecum* y *Allium sativa*, para aportar metionina (Met), mismas que fueron comparadas con alfalfa (*Medicago sativa*) y harina de soya extraída con solvente (*Glycine max*), como fuentes estándar de proteína, usando la técnica de producción de gas *in vitro* modificada para liberación de N-NH_3, estimando la cinética de fermentación y la digestibilidad de materia seca (72 h) en un diseño completamente al azar, con prueba de medias de Tukey. La concentración de N amoniacal de las mezclas herbales fue menor (P<0,05) que las fuentes de proteínas estándar, lo que indica que su proteína podría resistir la degradación ruminal. Los aditivos herbales con Lis o Met mostraron una concentración mínima de N-NH_3 en las primeras 4 h de incubación; a las 8 h la liberación, para dichos productos fue de 0,27 y 0,54 mg dL^{-1} respectivamente, menor (P<0,05) que la harina de soya extraída con solvente y alfalfa (1,15 y 2,24 mg dL^{-1} respectivamente).

Palabras clave

Allium sativa • Aditivo herbal • *Linum usitatissimum* • lisina • metionina • nitrógeno amoniacal • *Phaseolus mango* • proteína • rumen • *Trigonella foenum-graecum*

Introduction

Estimation of protein degradation for evaluation of feeds and calculation of the escape protein value of a particular protein, is necessary for formulating and meeting ruminant requirements (15, 39). Amino acids absorbed in the small intestine of ruminants derive from microbial protein and from dietary protein that escape ruminal degradation. In weaned young ruminants, a smaller rumen size (compared to the adult) limits dry matter intake and consequently microbial protein synthesis, resulting in the duodenal flow of microbial amino acids theoretically not being adequate to reach maximum growth potential (38, 39). Protein composition reaching the small intestine is also of great relevance given that microbial protein is considered limited in certain amino acids such as lysine, methionine and threonine, for growing ruminants (9, 47). Therefore, dietary undegradable rumen protein should contain amino acids that could complement the microbial contribution (38, 44).

Protein in plant products degrades extensively in the rumen. However, several herbaceous legumes such as *Phaseolus* spp and *Trigonella* spp have high concentrations of polyphenolic compounds (mainly tannins) and other secondary metabolites, that allow some protection from ruminal microorganisms. These tannins affect bacterial populations, improving ruminal fermentation efficiency, performing as natural feed additives (22, 25). These plants could increase amino acid absorption in the intestine. However, evidence about ruminal degradation and characterization of this type of species, is scarce. Therefore, the objective of the present study was to characterize two sources of herbal mixtures containing limiting amino acids (Lys and Met), analyze their chemical compositions, estimate ruminal protein degradation using ruminal ammonia concentration compared with known feeds as standard sources of protein (alfalfa and solvent extracted soybean meal) and characterize the kinetics of rumen dry matter degradation with the *in vitro* gas production technique.

Materials and methods

Two herbal mixtures were tested: one from *Phaseolus mango* and *Linum usitatissimum* (as contributor of herbal lysine; HL) and another from *Trigonella foenum-graecum* and *Allium sativa* (as herbal methionine; HM), in addition to alfalfa (*Medicago sativa*) and solvent extracted soybean meal (*Glycine max*), as standard sources of protein. The four protein substrates (HL, HM, alfalfa and solvent extracted soybean meal) were independently tested.

OptiMethionine and OptiLysine, corresponding to HM y HL respectively, were provided by Nuproxa Mexico (Indian Herbs and Nuproxa Switzerland). These feed plant additives are standardized products and have certified mixtures, following ISO 9001 and GMP (Good Manufacturing Practices).

Herbal products composition was determined using the methodology established by the A.O.A.C. (3): humidity percentage (method 934.01), crude protein by Kjeldahl method (N x 6.25) (method 954.01), ether extract (method 920.39) and ashes by calcination at 550 °C (method 923.03). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the Van Soest *et al.* procedures (1991).

In vitro gas production was determined according to Theodorou *et al.* (1994) modified in order to measure ammoniacal nitrogen. Rumen liquor was collected from a cannulated Holstein bull, about five years old and weighing 600 kg. Ruminal fluid was filtered through eight layers of cheesecloth and held under CO_2 in a water-bath at 39 °C. A 0.5 g sample was placed in 125 mL amber flasks. Gas pressure was measured at 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, 42, 48, 60 and 72 h of incubation, using a manual pressure gauge (scale 0 to 1 kg cm^{-2}). Gas pressure was then transformed into volume through the linear equation proposed by Ørskov and McDonald (1979). Gas production kinetics, namely maximum volume of gas produced (Vmax), Lag phase (L), gas production rate (S) and time required to reach half Vmax ($K_{0.5}$), were obtained using the logistic model of Pitt *et al*. (1999). *In vitro* dry matter digestibility (IVDMD) was estimated by filtering the residue from the flasks after 72 hours of incubation with a filter paper Waltham No. 41 and dried at 55 ° C for 48 h.

N-NH_3 concentration was determined by the *in vitro* gas production test (described above), extracting 1.2 mL of ruminal fluid from each incubated flask at 2, 4, 8, 12 and 24 h of incubation and mixing with 0.3 mL of metaphosphoric acid, kept at -18 °C. Later, the samples were centrifuged at 12,100 x g for 5 minutes in a Mini-spin eppendorf centrifuge. The supernatant was used to measure N-NH_3 concentration with the phenol-hypochlorite reaction (26). Readings were made at 630 nm with a spectrophotometer (Cary 100 UV-VIS). The results were multiplied by the sample dilution factor (4:1).

Incubation was performed twice. Each assay contained three replicates per tested substrate and their respective blanks. The experimental design was completely randomized with 4 treatments (HL, HM, solvent extracted soybean meal and alfalfa) and 6 repetitions per variable (Vmax, L, S, $K_{0.5}$, IVDMD, as well as the N-NH_3 concentration at 4, 8, 12 and 24 hours). Each flask used in gas production tests was considered an experimental unit. Data were analyzed with the JMP statistical software (42) and the means were compared with the Tukey test ($P< 0.05$).

Results and discussion

Chemical composition was similar between herbal mixtures (table 1, page 291). Considering that their formulation contains at least one legume (*P. mango* or *T. foenum-graecum*), crude protein percentage could have been higher than the value determined (20, 43, 44). However, the exact proportion in which these legumes and their plant parts are present in the herbal mixture, are unknown. Pinto *et al.* (2010) mentioned that forage chemical composition can be affected by specie, forage age, plant organ (fruits or leaves), growth site and soil type, among others. The tested herbal mixtures are certified by an amino acid formulation (lysine or methionine) analysis.

Table 1. Partial chemical composition of herbal mixtures.

Tabla 1. Composición química parcial de las mezclas herbales.

Item	Herbal lysine (HL)	Herbal methionine (HM)
Dry matter (%)	96.0	97.8
Organic matter (%)	92.6	85.0
Crude protein (%)	8.2	9.2
NDF (%)†	52.6	44.1
ADF (%)	33.1	25.9
Ether extract (%)	6.7	4.8
Calcium (%)	1.44	0.97
Phosphorus (%)	0.44	0.54
Methionine (g/100g protein)	1.74	5.23
Lysine (g/100g protein)	11.19	0.81
ME (Mcal kg^{-1}) ¶	2.57	3.02

† NDF: neutral detergent fiber, ADF: acid detergent fiber, ME: metabolizable energy. ¶ Estimated with *in vitro* digestibility equations (33) .

† NDF: fibra detergente neutro, ADF: fibra detergente ácido, ME: energía metabolizable. ¶ Estimada con ecuaciones de la digestibilidad *in vitro* (33) .

Kassi *et al.* (2000) explained that high concentrations of neutral detergent fiber (NDF) in forage are associated with lower intake, and high concentrations of acid detergent fiber (ADF) are associated with low ruminal digestibility, both undesirable characteristics reflected in the results of IVDMD in table 2. Legumes like *P. mango* have an advantage over grasses, because they mainly lignify their stems and not their leaves, as most grasses used for grazing. Therefore, greater stability in forage nutritional quality of woody legume species, is observed over time (7). These herbal mixtures are composed by finely ground herbs, therefore the effective NDF contribution is low, with minimal intake or digestion effects.

Legumes are considered a good source of protein, essential amino acids (such as lysine, leucine, isoleucine, phenylalanine and valine), unsaturated fatty acids (like linolenic and linoleic acids), dietary fiber, minerals (Ca, Fe and Zn) and vitamin C (4, 13, 14, 20), as well as various secondary metabolites. The species included in the mixtures evaluated, provide good metabolite diversity. The genus *Phaseolus* contains protease inhibitors, phytic acid (11) and polyphenols, as well as condensed tannins and flavonoids (anthocyanin glycosides: cyanidine, definidine and pelargonidine) (11, 20). *L. usitatissimum* contains polyunsaturated oils, mainly linolenic acid, as well as polyphenolic compounds, called lignans, with antioxidant activity (6, 12). The legume *T. foenum-graecum*, has antioxidant effects given by glutathione, *β*-carotenes and *α*-tocopherol, as well as tannins, alkaloids and saponins, particularly diosgenin (30, 50). Finally, *A. sativum* is a rich source of essential oils and organosulphurous compounds such as allicin, with specific antimicrobial activities. Total phenols, condensed tannins and essential oils present in this plant appear to be biologically active secondary metabolites, which modify rumen fermentation (22, 23, 34) and explain the resistance to protein degradation in the *in vitro* incubation.

Table 2. Kinetic parameters of ruminal fermentation and *in vitro* digestibility from herbal mixtures and protein sources.

Tabla 2. Parámetros de cinética de fermentación ruminal *in vitr*o y digestibilidad de mezclas herbales y fuentes proteicas.

Parameter	Source †				
	HL	HM	SM	A	SEM
$Vmax^{72}$ (mL g^{-1})	292.07^{b}	327.77^{ab}	340.67^{a}	290.43^{b}	7.541
S (h^{-1})	0.038^{c}	0.044^{ab}	0.039^{bc}	0.046^{a}	0.0012
L (h)	0.83^{c}	1.06^{c}	2.22^{b}	2.97^{a}	0.265
$K_{0.5}$ (h)	18.46^{a}	15.59^{bc}	17.47^{ab}	15.04^{c}	0.472
$IVDMD^{72}$ (%)	75.60^{d}	83.89^{b}	97.69^{a}	78.51^{c}	2.565

† HL: herbal lysine, HM: herbal methionine, SM: solvent extracted soybean meal, A: alfalfa, Vmax: maximum volume, S: fractional rate, L: Lag phase, $K_{0.5}$: time required to reach half of Vmax, IVDMD: *in vitro* dry matter digestibility, SEM: Standard error of mean. ^{abc} Means with different literals in the same row are different (P<0.05).

HL: Lisina herbal, HM: metionina herbal, SM: harina de soya extraída con solvente, A: alfalfa, Vmax: volumen máximo, S: tasa fraccional, L: fase lag, $K_{0.5}$: tiempo requerido para alcanzar la mitad de Vmax, IVDMD: digestibilidad *in vitro* de la materia seca, SEM: error estándar de la media. ^{abc} Medias con diferente literal en la misma hilera son diferentes (P<0,05).

Ruminal fermentation parameters are shown in table 2 (page 291). Solvent extracted soybean meal generated the highest gas production, probably due to the type of carbohydrates of the substrate since cellulose predominates in alfalfa and herbal mixtures. Soto *et al.* (1994) found that peptides and amino acids addition had no effect on ruminal fermentation parameters *in vitro* when bacteria is grown on cellulose-rich substrates. However, bacterial growth was stimulated with cellobiose or glucose. Although not statistically significant, gas production of herbal lysine was lower than that of herbal methionine, coinciding with Hernández *et al*. (2010), who explainedthat leaves with lower energy value generate less gas *in vitro*.

It has generally been observed that forages have a longer *lag* phase, especially when being of poor quality (2). However, although the herbal compounds analyzed in the present study have similar composition to that of any other forage, they have a very short *lag* phase, shorter than that determined for solvent extracted soybean meal and alfalfa, which can be explained by the proportion of its cellular contents.

Table 3 shows that N-NH_3 concentration achieved by herbal mixtures (HL and HM) was very low during the first 12 h of incubation compared to protein standards, remaining constant from the 4th hour of incubation. Raab *et al.* (1983) and Lorenz *et al*. (2011) reported that N-NH_3 concentration can be an estimator of protein degradation in the rumen. Therefore, these results would imply that part of the protein contained in these forages is resistant to rumen degradation.

Table 3. Ruminal N-NH_3 concentration (mg dL^{-1}) from herbal mixtures and protein sources incubated *in vitro.*

Tabla 3. Concentración ruminal de N-NH_3 (mg dL^{-1}) de mezclas herbales y fuentes proteicas incubadas *in vitro.*

Time h	Source †				
	HL	HM	SM	A	SEM
4	0.00^{c}	0.02^{c}	1.15^{b}	2.24^{a}	0.154
8	0.27^{b}	0.54^{b}	2.76^{a}	3.33^{a}	0.339
12	0.34^{b}	0.57^{b}	5.19^{a}	4.75^{a}	0.245
24	4.23^{b}	3.43^{b}	16.94^{a}	6.74^{b}	1.233

† HL: herbal lysine, HM: herbal methionine, SM: solvent extracted soybean meal, A: alfalfa, SEM: Standard error of mean. ^{abc} Means with different literals in the same row are different (P<0.05).

† HL: Lisina herbal, HM: metionina herbal, SM: harina de soya extraída con solvente, A: alfalfa, SEM: error estándard de la media. ^{abc} Medias con diferente literal en la misma hilera son diferentes (P<0,05).

Rates of rumen protein degradation depend on multiple factors. Bach *et al*. (2005) mention protein structure, interaction with other nutrients and proteolytic activity of the ruminal microbiota as key factors, among others. However, enzymatic inhibitors or anti-nutritional factors may influence protein degradation (27, 40). The most studied antinutritional factors are tannins, followed by secondary plant metabolites such as saponins, cyanogenic compounds, lecithins, alkaloids, oxalic acid and flavonoids (18, 19, 29). These factors can influence rumen protein degradation and synthesis, either by directly affecting the ruminal microorganisms, or by their interaction with nutrients (8, 22, 27).

Plants like *P. mungo*, *T. foenum-graecum* and *A. sativum*, contain significant amounts of condensed tannins, that react with proteins forming tannin-protein complexes through hydrogen bonds, hydrophobic interactions, ionic bonds and covalent bonds (41). These complexes can affect bacterial enzymes inhibiting carbohydrate and protein fermentation (16). In addition, tannins affect rumen proteolysis given that they can associate with soluble dietary proteins, protecting them from microbial action (8, 16, 41).

It has been reported that 100 g of *T. foenum-graecum* provides 4.63 g of saponins (1, 50). Hu *et al.* (2005) found that when the amount of saponins in an *in vitro* fermentation test is increased, the concentration of ammonia decreases significantly. Wang *et al.* (2000) also observed a decrease in N-NH_3 by including *Yucca schidigera* (a product rich in saponins), both *in vivo* (sheep feeding) and *in vitro*.

Various secondary metabolites have been identified when reducing the protozoan population. Total polyphenols, condensed tannins and saponins, were all reported as secondary metabolites of forages used in the herbal mixtures of the present study. Galindo and Marrarero (2005) reported the effect of *Leucaena leucocephala*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, *Sapindus saponaria* and other plants on rumen ciliated protozoa. Given that protozoa have high protein requirements, and ruminal N-NH_3 concentrations decrease with defaunation, this has been considered advantageous in low protein diets (31).

Decreasing rumen protein degradation of herbal mixtures (table 3, page 292) represents an advantage. Coomer *et al.* (1993) and De Almeida *et al.* (2015) statedthat supplementing young ruminants' diet with undegradable rumen protein, can increase protein and amino acid flow to the lower gastrointestinal tract and, therefore, increase the metabolizable protein supply. These herbal mixtures could be used as additives, not as protein supplement. To feed diets that maintain the concentration of N-NH_3 for microbial protein synthesis between 4 to 10.0 mg of N-NH_3/100 mL of rumen fluid, as well as to provide available energy for the ruminal ecosystem (41), is important. Ammonia values from the herbal mixtures in the first 12 h were below these values compared to the standard protein (table 3, page 292). Galindo and Marrarero (2005) mention that some legumes have soluble proteins, highly degradable in rumen, making it necessary to guarantee enough energy for adequate synthesis of microbial protein. Ruminal N-NH_3 concentration has been used to estimate rumen degradation (37) and lambs supplemented with herbal lysine have improved growth (28) confirming resistance to rumen degradation of the feed plant additive.

Conclusions

Ammonia concentrations found in the present study would imply that part of the protein contained in these forages is resistant to rumen degradation. Herbal mixtures evaluated could be used in ruminants feed as a source of undegradable rumen protein. However, the potential use as amino acid bypass for ruminants, needs further evaluation, in order to assess its degradation throughout the entire digestive tract.

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