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Comparison of a Polyherbal Mixture with a Rumen-Protected Lysine on Lamb Growth, Protozoan Count and Blood Chemistry

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Abstract
The objective of this study was to compare doses of a polyherbal mixture (Phaseolus mungo with Linum usitatissimum, OptiLysine® from Nuproxa México, Indian Herbs) and from rumen-protected lysine (RPL; AjiPro®-L) on lamb growth, changes in rumen protozoa and blood biochemistry. Fifty-six Pelibuey x East Friesian lambs (initial live weight 21.97 kg ± 4.29) were randomly assigned to treatments consisting of a control group, or three daily doses of lysine sources: polyherbal mixture (5, 10 and 15 g/d) and RPL (5, 10 and 15 g/d) dosed daily orally for 45 days. The lambs were fed individually with a basal ration (13.2% CP, 2.26 Mcal ME/kg) with an estimated duodenal flow in the basal diet of 6.45 g/d of lysine and 2.32 g/d of methionine. The doses of both sources did not affect the productive performance with the exception of the intake that showed a quadratic response with the RPL (p<0.05). The polyherbal mixture linearly stimulated the Entodinios population (p<0.10) and reduced the Holotrichs population (p<0.05), while the RPL linearly reduced the Holotrichs population (p<0.05). In blood biochemistry, the polyherbal mixture only affected lactic dehydrogenase (quadratic effect p<0.05) and RPL increased alkaline phosphatase (linear p<0.05) and decreased globulin (quadratic effect p<0.05). The results indicate that the polyherbal mixture and the rumen protected lysine did not improve the growth of the lambs, but the protozoan populations of the rumen were affected.
Key words: *Lambs, Lysine, Feed plant additive*

INTRODUCTION

Lysine and methionine have been recognized as growth limiting amino acids for young animals (NRC, 2007; Araújo et al., 2019) and are considered the most important in ruminant production (Flores *et al*., 2009; Prado *et al*., 2015; Mendoza *et al*., 2012). Rumen-protected lysine (RPL) has been supplemented to growing lambs with variable results (Araújo *et al*., 2019; Prado *et al*., 2015) and there are no precise estimations of lysine and methionine requirements as in dairy cattle (Lara *et al*., 2006; Vyas and Erdman, 2009), however, since lysine is an essential amino acid required for the synthesis of body proteins and is considered the first limiting amino acid in ruminant (Araújo *et al*., 2019) it is important to evaluate the available sources for growing lambs.
Since lysine and methionine have been recognized for optimal growth and milk synthesis in dairy cattle (Socha *et al*., 2005), sources of rumen protected methionine have been commercially available since several years ago, however, those of lysine are recent. The degree of protection of rumen bypass products has been estimated with *in situ* or *in vitro* techniques (NRC, 2007; Lara *et al*., 2003) but for RPL products there is little information available. Watanabe *et al*. (2003) estimated a ruminal bypass of 58.3% and Swanepoel (2010) reported a ruminal lysine bypass from 19 to 24%. It is assumed that the ingredients used to protect amino acids are inert

and do not affect ruminal microbiota, but some products have some compounds that include polyunsaturated fatty acids (Ardaillon *et al*., 1989; Ardaillon and Franzoni, 1992) that can affect rumen protozoa.
Herbal mixtures made with plants that have high concentrations of polyphenolic compounds (mainly tannins) and other secondary metabolites, can confer protection against degradation caused by ruminal microorganisms and supply essential amino acids. However, those metabolites could also affect the microbiota positively or negatively depending on the dose (Jouany and Morgavi, 2007). An experiment with growing lambs receiving a polyherbal mixture composed of *Phaseolus mungo* and *Linum usitatissimum* showed that intake, final weight and daily weight gain were improved as the dose was increased from 0 to 20 g/d (Mejía- Delgadillo *et al*., 2019).
Therefore, the objective of the present experiment was to compare a polyherbal mixture (*Phaseolus mungo* and *Linum usitatissimum*) and a rumen-protected source of lysine on lamb growth, blood parameters, and population changes of ruminal protozoa.

MATERIALS AND METHODS

The sources used were a polyherbal mixture elaborated with *Phaseolus mungo* and *Linum usitatissimum* (OptiLysine® from Nuproxa México, Indian Herbs) and the rumen protected lysine (RPL) was L-Lysine Monohydrochloride (AjiPro®-L from Animal Nutrition North America, Inc., Chicago). The experiment was conducted at the Centro de Enseñanza Práctica e Investigación en Producción y Salud Animal (CEPIPSA) of UNAM (CDMX, México) with 56 Pelibuey x East Friesian lambs (initial live weight 21.97 kg ± 4.29) that were randomly assigned to the treatments consisting of a control group, or three daily doses of the polyherbal mixture (5, 10 and 15 g/d) or the RPL (5, 10 and 15 g/d) for 45 days, fed individually with a basal diet (dry base) containing: corn grain (34.0%), corn stover (55.3%), cane molasses (6.0%), urea (2.7%) and mineral premix (2.0%), with 2.26 Mcal ME/kg and 13.2% crude protein (CP). A duodenal flow of 6.45 g/d of lysine and 2.32 g/d of methionine was estimated for the basal diet. The doses of the polyherbal and RPL mixture were administered daily individually in bolus elaborated with a mixture of 750 g of corn flour, 50 g of molasses and 100 mL of water (Alonso-Mélendez *et al*., 2016). Before starting the experiment, the lambs were dewormed, bacterinized and had an adaptation period of 15 days to the experimental diet.
On day 45 of the experimental period, a ruminal fluid sample was taken using an esophageal tube prior to feeding; once the ruminal fluid was collected, it was filtered through 8 layers of gauze. The samples were diluted with 200 mL/L of glycerol, stained using Lugol's solution 1:1 (D'agosto and Carneiro, 1999) and kept under refrigeration for the subsequent microscopic count. After staining (minimum 4 hours), pipetting was performed for counting, in a Sedgewick-Rafter chamber (Dehority, 1984).
Samples of the polyherbal mixture were used to determine the lysine concentration by Ultra Performance Liquid Chromatography (UPLC) in the Chemistry and Food Analysis Laboratory of the Faculty of Chemistry of UNAM. The lysine content was 11.19 g of Lys/100 g of protein. The RPL reported in technical data is 50% lysine, 49% fat and 1% soy lecithin on a dry basis.
At the end of the experimental period, approximately 5 mL of blood was collected from each lamb by jugular venipuncture. These samples were centrifuged at 3000 rpm for 10 minutes, to separate the serum which was extracted with a micropipette and stored at -5 °C, for subsequent laboratory analysis. The concentrations of glucose, urea, uric acid, cholesterol, total protein, albumin, globulin, bilirubin, creatinine, alkaline phosphatase (ALP), lactic dehydrogenase (DHL), aspartate aminotransferase (AST), calcium and phosphorus were determined using an autoanalyzer Kontrolab 2017.

Statistical analysis

A completely randomized experimental design with eight replications per treatment was used. The linear or quadratic response of each source of lysine was tested with orthogonal polynomials (Mirman, 2014). The initial weight was used as a covariate for the variable's final weight, average daily gain (ADG), dry matter intake (DM) and feeding efficiency, in addition to blocking by sex.

RESULTS AND DISCUSSION

The doses of both sources did not affect the productive performance with the exception of the intake that showed a quadratic response with the RPL ($p<0.05$; Table 1) although numerically showed that ADG could be increased by 5% using 10 g of the polyherbal mixture and in 7% with 15 g of RPL over the control group.

Table 1. Effect of the type and daily dose of lysine on the growth of 45-day-old lambs.

Product	Product doses g/d					*P value*	
	0	5	10	15	SD	Linear	Quadratic
Polyherbal mixture*							
Final weight (kg)	29.8	30.55	31.0	29.1	5.20	0.96	0.81
ADG (kg)	0.177	0.173	0.186	0.171	0.0398	0.96	0.81
Intake DM (g/d)	910.0	854.5	871.3	854.2	162.36	0.37	0.33
Feed efficiency	0.142	0.138	0.159	0.147	0.0252	0.58	0.40
Rumen-protected Lysine							
Final weight (kg)	29.8	28.4	31.3	30.4	5.89	0.73	0.44
ADG (kg)	0.177	0.170	0.174	0.190	0.0521	0.73	0.44
Intake DM (g/d)	910.0	831.9	881.2	956.8	222.81	0.80	0.04
Feed efficiency	0.142	0.152	0.142	0.144	0.0213	0.90	0.70

SD: standard deviation; DM: dry matter; ADG: average daily gain. Initial weight was used as a covariate. * Elaborated with *Phaseolus mungo* and *Linum usitatissimum.*

Protein evaluations in growing ruminants at different doses allow the estimation of efficiency of protein utilization (Nakamura *et al*., 1994) and are conducted with low energy content, however, a study with labeled lysine [U-13C] showed that the incorporation of the amino acid into the muscle is related to the energy intake (Savary *et al*., 2001) which could limit the response in this study, because of the same herbal mixed improved weight gain in lambs until 10% with higher energy content (Mejía-Delgadillo *et al*., 2019), greater than the 5% observed with the 10 g in this experiment.
There are few studies in sheep with ruminally protected amino acids, but the information in cattle suggests that in growing animals methionine may be the main limiting amino acid and lysine the second (Hussein and Berger, 1995; Zinn *et al*., 2007). Information from growing Awassi lambs suggests that they may require more methionine in the final stage of the finishing period (Abdelrahman *et al*., 2003). However, a study with sources rich in lysine suggests that this is not necessarily true (Ponnampalam *et al*., 2005), therefore it is necessary to estimate the duodenal flows of lysine and methionine to explain the response in each experiment.
A possible cause for the lack of response in both sources could be that the relationship between lysine and methionine was altered as suggested in evaluations of protected methionine in lambs (Acosta *et al*., 2012; Rodríguez-Guerrero *et al*., 2018). In estimates for experiments on growing sheep with average gains of 274 g/d, the duodenal fluxes of lysine and methionine on average were 10.07 and 2.25 g/d respectively; lambs from this experiment supplemented with the polyherbal mixture had an average flow estimated of 6.63 g/kg of lysine and 2.87 of methionine, while for those supplemented with RPL, were 10.27 and 2.30 g/kg of lysine and methionine, respectively.
Another possibility for lack of response is that the digestibility of lysine in the intestine has been low, but the information on the products evaluated is scarce. Watanabe *et al*. (2003) estimated an intestinal digestibility of 49.5% for another commercial lysine rumen-protected, estimating that it only provided 28.9% metabolizable lysine. It is possible that the intestinal digestibility of the RPL has not been a limitation based on the productive response of growing pigs fed with the same RPL (Figueroa *et al*., 2019). The polyherbal mixture data does not indicate that it has been a limiting factor for the growth of lambs (Mejía-Delgadillo *et al*., 2019).

Prado *et al*. (2015) included another source of RPL (1 g/d) and did not observe a response in the ADG, but a decrease of 8.7% in intake, a greater effect than that observed in this study (5.4%) with the herbal lysine. On the contrary, Araújo *et al*. (2019) observed a linear increase in intake (16.9%) but their RPL provided bypass methionine with a ratio of lysine methionine of 3:1. It is known that amino acids can stimulate intake through the ghrelin (Sugino *et al*., 2010) and that the imbalance can negatively affect intake (Harper *et al*., 1970).

The polyherbal mixture linearly stimulated the Entodinomorph population ($p<0.10$) but reduced the number of Holotrichs ($p<0.05$) while the RPL only reduced the Holotrichs (linear, $p<0.05$) (Table 2). It is assumed that the mechanisms of action in the protozoa were different due to the characteristics of the products (herbal versus synthetic).

Table 2. Effect of type and a daily dose of lysine on ruminal protozoa in growing lambs.

Product	Product doses g/d					*P value*	
	0	5	10	15	SD	Linear	Quadratic
Polyherbal mixture*							
Entodinidae	6544	6695	8490	8189	3679.1	0.06	0.94
Holoreichidae	243	144	167	125	136.3	0.02	0.12
Total	6787	6839	8656	8314	3674.5	0.07	0.89
Rumen-protected Lysine							
Entodinidae	6544	7842	6529	6773	3063.6	0.90	0.53
Holoreichidae	243	249	135	130	153.5	0.01	0.82
Total	6787	8091	6665	6703	3133.2	0.99	0.54

SD: standard deviation. * Elaborated with *Phaseolus mungo* and *Linum usitatissimum.*

Linum usitatissimum has pectins and mucilage (Saastamoinen and Särkijärvi, 2020) that are fermented by bacteria and protozoa, *Linum usitatissimum* seed oil contains fatty acids (omega-3) (Cunnane *et al*., 1993) and unlike others herbal plants, does not have high antioxidant metabolites (Szerlauth *et al*., 2019), but it is not clear which component could have reduced the numbers of Holotrichs. *Linum usitatissimum* seed is used by farmers to treat diarrhea in calves, and other digestive problems, and is known by its anti-inflammatory, antidiarrheal, and spasmolytic properties related to the content of mucilaginous polysaccharides (Mertenat *et al*., 2019). The other plant in the polyherbal product is the legume *Phaseolus mungo* that has been characterized as a protein ingredient (Sobha and Susan, 2014) but also has functional properties derived from its content of oligosaccharides and sugars (Kavitha *et al*., 2013); the latter stimulate the presence of protozoa. It is possible that the reduction in the Holotrichs has been caused by the chemotaxis to the sugars that stimulate their migration towards the liquid phase in the rumen (Diaz *et al*., 2014), which could explain the decrease in this population. The increase of Entodinios with sugars has been previously reviewed and explains the response of these ciliates (Ortega and Mendoza, 2003).

The effects of RPL can be explained by the fraction of the product that breaks down in the rumen. Ruminally protected products are considered to be inert in the rumen, but they have a variable fraction that degrades in the rumen (Lara *et al*., 2003; Watanabe *et al*., 2003). Some commercial RPL products use lysine sulfate, hydrogenated fat, oleic acid, and ethyl cellulose, so partial release of these compounds can have rumen effects, such as those seen in the ciliates. Han *et al.* (1996) reported higher concentrations of ammonia N with the inclusion of RPL confirming that the products are not completely protected. It is not clear what specific RPL compound reduced Holotrichs.

The polyherbal mixture had no effect on blood biochemistry with the exception of lactic dehydrogenase (DHL, quadratic effect p<0.05), while RPL increased albumin (linear p<0.10) and globulin showed a quadratic response (p<0.05), which affected the albumin/globulin ratio (linear, p<0.05; quadratic p<0.01). RPL caused an inverse quadratic effect (p <0.05) on bilirubin and a positive on lactic dehydrogenase (quadratic, p<0.10).

The main serum indicators used to evaluate liver disorders include AST, ALT, bilirubin, total proteins, and albumin (Dufour *et al*., 2000), but they must be evaluated integrally and the results do not show that the sources of lysine have affected the liver functionality. High levels of DHL with the 5 g/d dose of the polyherbal mixture could be an indicator of a high rate of turnover of muscle synthesis (Beebee and Carty, 1983) and not a response to compounds present in *Linum usitatissimum* as cyanogenic glycosides (Saastamoinen and Särkijärvi, 2020) that would have caused a linear response in liver enzymes.

The RPL did not cause changes in metabolites associated with protein metabolism (total protein, urea, and creatinine), and changes in albumins or globulins were minor (Table 3). Albumin is an indirect nutritional indicator related to amino acids and globulins give information about the immune system; therefore, the changes were not indicative of health problems (Russell, 2007). Total bilirubin only increased with 15 g/d of RPL and the linear response in alkaline phosphatase (Table 3) suggests that some of the compounds used to coat the lysine may be causing minor liver stress.

Table 3. Effect of the type and daily dose of lysine on the blood biochemistry of growing lambs.

Product	Product doses g/d				SD	*P value*	
	0	5	10	15		Linear	Quadratic
Polyherbal mixture*							
Glucose mg/dL	87.1	83.2	92.1	92.3	13.66	0.18	0.77
Urea mg/dL	31.1	30.4	28.8	34.1	6.60	0.39	0.35
Uric acid mg/dL	0.68	0.60	0.61	0.76	0.306	0.61	0.52
Cholesterol mg/dL	80.4	108.9	81.1	83.8	25.21	0.73	0.24
Total protein g/dL	6.6	6.5	6.8	6.6	0.58	0.72	0.40
Albumin g/dL	3.5	3.3	3.5	3.5	0.48	0.61	0.88
Globulin g/dL	3.1	3.3	3.2	3.1	0.40	0.98	0.48
A/G relation	1.12	1.0	1.09	1.06	0.197	0.90	0.84
Bilirubin mg/dL	0.48	0.50	0.48	0.44	0.147	0.29	0.31
Creatinine mg/dL	1.04	1.03	1.09	1.06	0.143	0.40	0.87
ALP U/L	65.0	65.1	59.7	55.1	20.08	0.33	0.53
LDH U/L	100.9	124.4	105.4	101.9	31.49	0.46	0.04
AST U/L	18.3	17.3	13.5	14.3	9.53	0.28	0.61
Calcium %	9.4	9.5	9.8	9.3	1.00	0.93	0.47
Phosphorus %	4.7	4.8	4.5	4.8	0.59	0.65	0.99
Rumen-protected Lysine							
Glucose mg/dL	87.1	87.0	88.6	96.0	16.11	0.29	0.67
Urea mg/dL	31.1	29.4	30.0	30.5	4.91	0.93	0.90
Uric acid mg/dL	0.68	0.84	0.66	0.64	0.317	0.41	0.39
Cholesterol mg/dL	80.4	82.5	99.4	98.8	23.19	0.30	0.70
Total protein g/dL	6.6	6.5	6.9	6.6	0.65	0.65	0.32
Albumin g/dL	3.5	3.4	3.7	3.8	0.61	0.08	0.72
Globulin g/dL	3.1	3.5	3.3	2.9	0.49	0.17	0.05
A/G ratio	1.12	0.98	0.94	1.44	0.379	0.03	0.01
Bilirubin mg/dL	0.48	0.44	0.44	0.58	0.153	0.13	0.05

Creatinine mg/dL	1.04	1.03	0.89	1.01	0.165	0.56	0.15
ALP U/L	65.0	72.7	78.6	95.6	25.73	0.05	0.54
LDH U/L	100.9	128.3	117.4	106.8	31.86	0.28	0.06
AST U/L	18.3	21.8	15.8	17.0	12.59	0.51	0.99
Calcium %	9.4	8.9	9.0	9.5	0.96	0.94	0.31
Phosphorus %	4.7	4.4	4.5	4.6	0.53	0.80	0.35

SD: standard deviation; ALP: alkaline phosphatase, LDH: lactic dehydrogenase, AST: aspartate aminotransferase. * Elaborated with *Phaseolus mungo* and *Linum usitatissimum*

The adequate and balanced supply of lysine and methionine is important to achieve the maximum potential of growing lambs and there is information that shows that protein supplementation with bypassing amino acids, particularly methionine and lysine, can improve intake, growth, reproductive efficiency and milk production. It is possible that in rations with higher energy content, the beneficial effects of the products used in this study could be manifested.

CONCLUSIONS

The results indicate that the polyherbal mixture with *Phaseolus mungo* and *Linum usitatissimum* and commercial rumen-protected lysine did not improve lamb growth, but had an effect on rumen protozoan populations presumably by different mechanisms of action.

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