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Ruminants

Effect of *Astragalus mollissimus* on Ruminal Fermentation, Methane Production and Performance of Sheep

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ABSTRACT

Recent studies have shown the anti-methanogenic capacity of *Astragalus mollissimus* (AM), a plant found in semiarid environments, which is known to produce 3-nitro-1-propionic acid (3NPA) and 3-nitropropanol (3NPOH). However, little is known about the effects of direct supplementation in basal diets, given that it is also known to cause cattle poisoning by nitro toxins in rangelands. In the present study, two experiments were carried out to determine CH_4 and volatile fatty acid production, animal performance and the presence of nitrocompounds in blood. In Experiment 1, four Pelibuey sheep (BW 52.8 ± 6.05 kg) were assigned to a 4 × 4 Latin square arrangement. In Experiment 2, 20 Dorper sheep were randomized to five treatments. In both experiments, AM was supplemented and fully homogenized into diets consisting of 67% oat hay and 33% concentrate. The supplementation with different amounts of AM reduces ($p \leq 0.05$) the total gas and methane production. Methane was reduced by 60% when 1 g AM kg^{-1} BW day^{-1} was supplemented. No effects ($p > 0.05$) were observed in feed consumption and average daily gain. However, feed conversion was increased ($p < 0.05$) with AM supplementation. Finally, no differences ($p > 0.05$) were observed in nitrocompound concentration in plasma. These results demonstrate that 3NPA and 3NPOH from biological sources possess desirable anti-methanogenic properties to be considered supplementation alternatives.

1 | Introduction

Unlike humans and other monogastric animals, ruminants can digest plant fibre through ruminal fermentation, converting the end products into useful products such as milk and meat (Ungerfeld 2018). During enteric fermentation large amounts of hydrogen (H_2) and carbon dioxide (CO_2) are released, potentially increasing the ruminal partial pressure if accumulated. Therefore, their disposal through ruminal methanogenesis, which uses these molecules as the main substrates, is crucial to maintain an adequate partial pressure in rumen (Schink 2006). Consequently, H_2 and CO_2 clearance is closely related to the production of methane (CH_4) (Hungate et al. 1970; Hobson and Stewart 1997; Duin et al. 2016). Thus, despite the impact on the environment of the greenhouse gases (GHGs) generated during this process, mainly methane and carbon dioxide (Ungerfeld 2018), methanogenesis is an essential pathway.

Although CH_4 is the second most abundant GHG (0.97 ± 0.16 W m^{-2}) in the atmosphere, followed by CO_2,

its warming potential is 28 times higher than that of CO_2 (Myhre et al. 2013), reaching up to 86 times higher in 20 years (IPCC 2018). Agriculture accounts for about 11% and 13% of CO_2 and CH_4, respectively, of the total GHG (Hristov et al. 2013). The methane produced by the enteric fermentation of ruminants is the main anthropogenic source of CH_4, releasing between 1.6 and 2.7 GtCO2e (Herrero et al. 2016), which reaches about 3.3% of the total anthropogenic GHG (IPCC 2018). On the other hand, ruminal methanogenesis uses up 5%–12% of the gross energy consumed by the ruminant, which is a loss of energy (Johnson and Johnson 1995). Thus, in vitro and in vivo studies are needed to find nutritional alternatives, feedstuffs or additives capable of reducing CH_4 emissions into the environment without compromising the appropriate ruminal fermentation and animal production.

In this regard, several alternatives have been evaluated, some of them including chemical interventions through the use of nitrocompounds. Some nitro compounds, such as nitrooxypropanol (3NOP), nitroethane, 2-nitroethanol, 2-nitro-1-propanol, and 3-nitro-1-propionic acid (3NPA) have been shown to be capable of inhibiting ruminal CH_4 production under in vitro conditions (Anderson et al. 2003; Anderson et al. 2008). The main mechanisms of action proposed for the decrease in CH_4 production by nitrocompounds are the inhibition of the methyl coenzyme M reductase (MCR) (Duin et al. 2016) and the diversion of the flow of reducing substrates, notably hydrogen, necessary for methanogenesis (Teng and Kim 2021). This redirection of substrates leads to β-alanine production when 3NPA acts as an alternative electron acceptor (Ochoa-García et al. 2019). Ochoa-García et al. (2019) evaluated 3NPA as a feed supplement under in vitro conditions. They found a strong reduction in CH_4 production; however, total production of volatile fatty acids (VFAs) was decreased up to 37% when the dose on 3NPA was increased. Therefore, the supplementation with 3NPA could negatively affect the production under in vivo conditions. Additionally, in vivo studies have shown that nitroethane and 2-nitro-1-propanol can reduce the production of enteric CH_4 (Anderson et al. 2006; Gutierrez-Bañuelos et al. 2007). Despite having reported positive results in the reduction of CH_4 production, their high costs and synthetic origin are limiting factors. Moreover, the dose and degree of toxicity of these nitrocompounds need to be addressed (Ochoa-García et al. 2019).

Currently, research is focused on natural alternatives to reduce CH_4 production, reduce costs, avoid toxicity towards the animal and avoid the presence of residues in animal derivative food products. In this regard, the use of plant nitrocompounds can be a suitable alternative. The capability of *Astragalus* spp. to produce nitrogenous derivatives was first noted by Stermitz et al. (1969); Stermitz et al. (1972) reported the presence of nitrocompounds miserotoxin, cibarian, karakin, hiptagin, 3-nitropropanol (3NPOH) and 3-nitropropanoic acid (3NPA). Moreover, the acid hydrolysis of the side chain of miserotoxin produces 3NPOH, whereas the 3NPA is produced by acid hydrolysis of cibarian, karakin and hiptagin.

More recently, Ochoa-García et al. (2021) found that *Astragalus mollissimus* (AM) extract reduced CH_4 production as much as 98% when the plant extract was supplemented to ruminal microbes under in vitro conditions. Nevertheless, as is known, AM produces 3NPA, which is a potent neurotoxic compound responsible for causing severe selective striatal lesions when it was administrated to baboons in doses of 33 mg kg^{-1} d^{-1} (Palfi et al. 1996). Therefore, in order to guarantee safety for humans and animal welfare, it is essential to know the appropriate dose of supplementation, their effects on the animal performance and the quantification of residual metabolites of these nitro compounds in plasma.

Thus, this study aimed to quantify CH_4 and VFA production, animal performance and the presence of nitrocompounds in the blood of sheep consuming different doses of AM as a natural source of nitrocompounds.

2 | Materials and Methods

2.1 | Plant Material Collection

The plant collection of AM was carried out manually during the spring of 2019 in the surrounding area of Chihuahua City, Mexico. The collected material was dried at 65°C for 48 h in a forced air oven (Imperial II incubator; Chicago Surgical & Electrical Co., Div. Lab-Line Instruments INC; USA) and subsequently ground to achieve a homogeneous particle size of 2 mm.

2.2 | Animals, Experimental Design, Diets and Housing

Two experiments were carried out.

2.2.1 | Experiment 1

For the total gas (TG) and methane (CH_4) production trial, four ($n = 4$) 10-month-old F1 Dorper × Pelibuey male sheep were used (BW = 52.8 ± 6.05 kg). Sheep were randomly assigned into a 4 × 4 Latin square arrangement and kept in individual metabolic chambers where they were fed daily at 09:00 h with a basal diet (Table 1) formulated following the recommendations for meat sheep (NRC 2007). The diet was offered at a dose of 1340 g day^{-1} with a forage/concentrate ratio (33:67), and residual feed was collected daily. Treatments were designed to contain 0, 0.25, 0.50 and 1.00 g of AM kg^{-1} BW day^{-1}, which was manually mixed with the basal diet. Animals had 2 weeks of diet adaptation, then the respective treatment was given to them during 4 days in a row, leaving the three subsequent days for elimination of the residual nitrocompound. On Day 5 of each period, samples of 100 mL ruminal fluid were collected at 10:00 AM according to the method described by Paz et al. (2016), with some adaptations. Ruminal fluid samples were collected using a manual extraction pump with a tube of 18 mm diameter and 80 mm length through the fistula of the sheep (Nalgene repairable Hand Vacuum Pumps; Thermo Fisher Scientific INC, China). The samples were used to study concentrations of CH_4 and VFAs. The ruminal fluid was immediately sent to the laboratory, where it was aliquoted into glass tubes with 10 mL of the sample each. The tubes were then sealed with a rubber stopper and aluminium ring to preserve the anaerobic conditions. The samples for gas composition analysis were incubated for 24 h at 39°C with constant shaking at 110 rpm.

TABLE 1 | Ingredients and chemical composition of forage, basal diet and experimental concentrate.

	Inclusion in feed (%)					
	AF	**DM**	**ME**	**CP**	**Ca**	**P**
Alfalfa hay	30.5	27.45	0.68	5.64	0.43	0.06
Corn flaked	36.1	32.12	1.17	3.61	0.01	0.1
Dried distilled grain	10	9.2	0.31	2.7	0.033	0.055
Soybean meal	13	11.57	0.40	6.48	0.039	0.08
Molases	5.3	3.97	0.13	0.30	0.053	0.005
Calcium carbonate	1	1	0	0	0.39	0
Magnesium oxide	0.7	0.7	0	0	0	0
Salt	0.8	0.8	0	0	0	0
Minerals	0.9	0.9	0	0	0.108	0.09
Palm oil	1.7	1.68	0.11	0	0	0
Total			**2.84**	**18.74**	**1.06**	**0.41**

Abbreviations: AF, as feed; Ca, calcium; CP, crude protein; DM, dry matter; ME, metabolizable energy; P, phosphorus.

The samples to analyse VFAs and nitrocompounds were directly frozen at −20°C until analysed.

2.2.2 | Experiment 2

For the animal performance and blood metabolites trial, 20 male F1 Dorper × Pelibuey sheep, 120 days average age and 29.0 ± 3.0 kg of live weight, were randomly assigned to one of the five treatments. A dose of 0.75 g of AM kg^{-1}BW day^{-1} was included as an additional treatment in this experiment along with the ones used in Experiment 1. Four animals were additionally assigned to each treatment. Treatments were as follows: T1–T5 (0.0, 0.25, 0.50, 0.75 and 1.00 g of AM kg^{-1} BW day^{-1}, which was manually mixed with the basal diet). The basal diet was fed as 1340 g $(a\ day)^{-1}$ with a forage/concentrate ratio (33:67), and the rejected feed was collected daily. Alfalfa hay was used as a forage source. Animals were fed twice daily (08:00 and 16:00 h) and allowed to drink fresh water at any time. Prior to the experiment, animals were dewormed using the product Panacur following the dosage and indication of the manufacturer (MSD Animal Health, Rahway, New Jersey, USA).

The experimental phase was 60 days long, starting with a 14-day adaptation period, followed by 46 days of supplementation and sample collection. Live weight was recorded at Days 1, 15, 30, 45 and 60. Feed consumption (FC) was measured daily. Average daily gain (ADG) was obtained by subtracting the earlier weight from the actual one and dividing it by the number of days of the specific period. The feed conversion ratio (FCR) was expressed as FC per unit of body weight gain. During the experiment, physiological variables (body temperature, respiratory rate, heart rate and panting) were assessed by a certified veterinarian. On Days 30 and 60, blood samples were collected via jugular puncture using heparinized vacuum tubes for later quantification of nitrocompound concentration in plasma.

2.3 | Laboratory Analyses

2.3.1 | Determination of TG Production and Composition

After 24 h of incubation, TG production was determined by measuring the headspace pressure in all the tubes (Theodorou et al. 1994). This measurement was carried out using a FESTO pressure transducer (SIEMENS, Munich, Germany).

Gas composition was determined by gas chromatography according to Allison et al. (1992). The procedure was carried out using a GOW-MAC Series 580 gas chromatograph, fitted with a Carbosphere 80/100 packed column, 5682 PC, which was calibrated, and a standard curve previously calculated. Nitrogen was used as a carrier gas at a flow of 20 mL min^{-1}.

2.3.2 | Determination of VFAs

VFA production was determined by gas chromatography following the procedure of Galyean (1989). The ruminal fluid samples were centrifuged, and 5 mL of the supernatant was collected and mixed with 1 mL of 25% metaphosphoric acid and an internal standard of 2-ethyl butyric acid, kept for 30 min in ice water and subsequently centrifuged at 10,000 × *g*. The standards for the calibration curve were prepared in the same way. A PerkinElmer Clarus 400 gas chromatograph equipped with a 30 m long Poropak-Q stainless steel capillary column, with helium as a carrier gas, was used at a flow of 20 mL min^{-1}.

2.3.3 | Determination of Nitrocompounds in Plasma

The nitrocompound (3NPA and 3NPOH) quantification in plasma samples was carried out according to the procedure described by Muir and Majak (1984). Briefly, heparinized whole blood was low-speed centrifugated (7000 rpm, 10 min), then 2 mL of plasma were obtained and treated with cold 0.6 N perchloric acid (1 mL) and chilled at 0°C for 30 min. The sample was centrifugated at 40,000 × *g* for 15 min, and the supernatant was recovered for HPLC analysis. Standards of 3NPA and 3NPOH for the calibration curves were prepared in the same way. 3NPA and 3NPOH were eluted isocratically using 0.15% orthophosphoric acid as a mobile phase at 2.0 pH and a flow of 0.9 mL min^{-1} with a UV-100 variable wavelength detector set at 210 nm.

2.4 | Statistical Analysis

TG and methane (CH_4) production per treatment in Experiment 1 were assessed by linear mixed-effects models. The models incorporated treatment as a fixed effect and both animal and week as random effects.

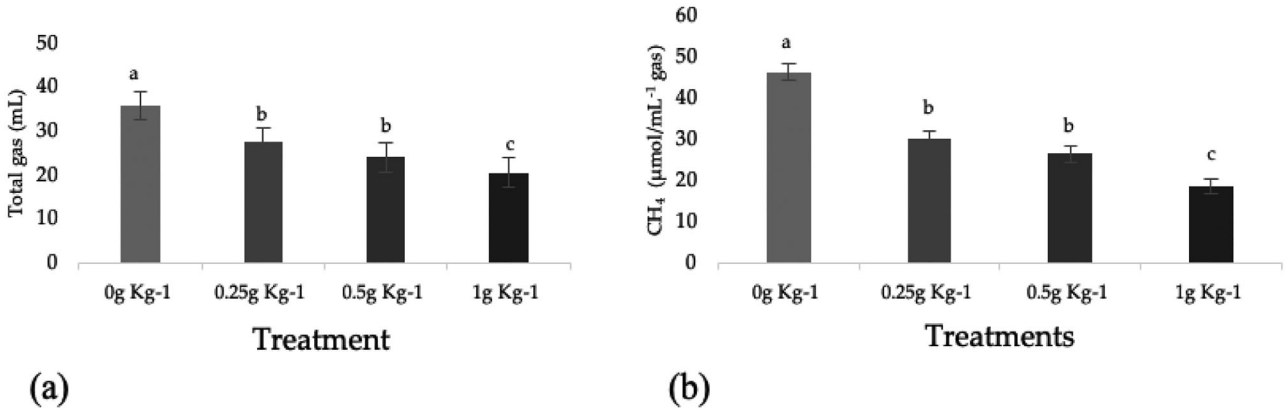

FIGURE 1 | Total gas (a) and methane (b) produced by rumen microbes after 24 h of incubation with different doses of *Astragalus mollissimus*. Values are least squares mean ± standard error. Different letters indicate significant differences ($p \leq 0.05$).

For each response variable, analysis of variance (ANOVA) was performed to evaluate the significance of the treatment effect. Following the ANOVA, post hoc pairwise comparisons were conducted to identify specific differences between treatment levels.

All statistical analyses were conducted using R (version 4.2.3) and RStudio (version 1.4.1106). The lme4 package was used for modelling, the car package for ANOVA, and the emmeans package for post hoc comparisons. A significance level (alpha) of 0.05 was applied for all statistical tests.

In Experiment 2, average daily weight gain (ADG), dry matter intake (DMI) and feed conversion ratio (FCR) were analysed using the general linear model (GLM) procedure in SAS. The model accounted for treatment as class fixed effects and birth and actual (at the beginning of the trial) body weight as linear regression effects.

3 | Results and Discussion

3.1 | Experiment 1

TG production was reduced as the dose of AM increased in the feed (Figure 1a), supporting the results observed by Ochoa-García et al. (2021) in the in vitro experiments with AM. No differences were observed between dosages of 0.25 and 0.50 kg of BW.

Methane production was reduced in tubes supplemented with AM following the same tendency as TG production (Figure 1b). These results are also in line with those found by Ochoa-García et al. (2019), thus suggesting the dose-dependent effects of the nitrocompounds, in which reductions were 35%, 43% and 60% for 0.25, 0.50 and 1.0 g kg^{-1} BW day^{-1}, respectively. As can be noted, the high dosage had the strongest effect in the reduction of methane compared to the control. Similar data were reported by Alemu et al. (2021), who investigated the use of 3-nitooxypropanol under in vivo conditions to reduce methane emissions from beef cattle in a feedlot. They also found a dose-dependent effect.

The supplementation of AM did not affect the production or composition of VFAs ($p > 0.05$). Data are shown in Figure 2. These data support the behaviour observed in the animal performance trial. However, the VFAs here observed differ from those reported by Ochoa-García et al. (2019), who observe a decrease in VFA production when 3NPA was supplemented. As is known, the AM plant contains 3NPA; however, it is not the only nitrocompound in that plant. Moreover, it is possible that the synthetic origin of the NPA used by Ochoa-García et al. (2019) had a strong effect on microbial populations.

3.2 | Experiment 2

Animals receiving the maximum dosage (1.0 g of AM kg^{-1} BW day^{-1}) showed some signs of intoxication, such as red eyes and dry mucus in the nasal cavities, as well as reduced activity. As the experiment progressed, these signs became more obvious, suggesting that the animals were developing several levels of methaemoglobinaemia, according to the findings of Shiono et al. (2001) for animals consuming some plants containing nitrocompounds. Additionally, it is suspected that 3NPA inhibits the succinate dehydrogenase (SDH) activity, disrupting the energy production by the cells, resulting in the reduced activity observed (Abdalla et al. 2019). No signals of intoxication were observed for the rest of the treatments.

DMI was not affected ($p < 0.05$) by supplementation (Table 2). Our findings support those by Abdalla et al. (2019) for the inclusion of *Astragalus membranaceus* in the diet of sheep. They did not observe differences between DMI in supplemented and unsupplemented sheep, probably as a result of the good integration of *Astragalus* into the diet, avoiding or reducing the selectivity of the sheep. However, Stegelmeier et al. (1999) reported a reduction in FC on Day 16 when ewes were supplemented with 0.4 g of AM. On the contrary, Wang et al. (2021) reported that the addition of *Astragalus* to the diet increased the consumption of dry matter. They suggest this behaviour is due to the fact that AM did not modify the palatability of the diet, even when a slight selection was observed.

The results show that AM supplementation had no effect ($p > 0.05$) on ADG (Table 2). Our findings differ from those reported by Stegelmeier et al. (1999), who reported that sheep supplemented with 0.4 g of locoweed (*Oxytropis sericea*) per day

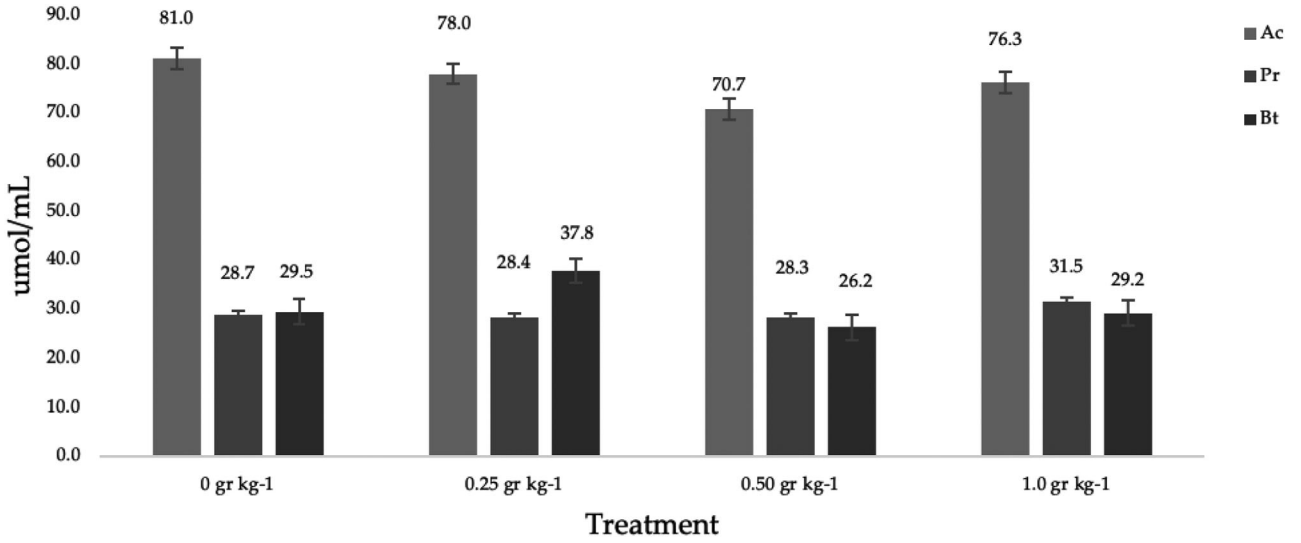

FIGURE 2 | Effect of different treatments of *Astragalus mollissimus* supplementation on the volatile fatty acids production and composition. Ac, acetic acid; Bt, butyric acid; Pr, propionic acid.

TABLE 2 | Least square means (±standard error) for animal performance of lambs supplemented with different amount of *Astragalus mollissimus*.

Treatment (g kg BW)	Initial body weight (kg)	Final body weight (kg)	ADG (kg)	DMI (kg)	FCR (kg)
0	31.1 ± 2.39	42.4 ± 2.9	0.333 ± 0.049	1.58 ± 0.047	4.82 ± 0.786[a]
0.25	30.6 ± 2.39	40.2 ± 1.65	0.280 ± 0.032	1.62 ± 0.073	5.87 ± 0.876[ab]
0.50	31.8 ± 3.65	40.7 ± 3.75	0.249 ± 0.029	1.65 ± 0.059	6.80 ± 0.852[b]
0.75	31.3 ± 6.04	42.3 ± 5.27	0.324 ± 0.049	1.67 ± 0.045	5.24 ± 0.771[ab]
1.00	35.9 ± 4.47	44.4 ± 3.5	0.287 ± 0.06	1.66 ± 0.084	5.90 ± 0.872[ab]
p value	NA	NA	0.117	0.156	0.038

Note: Means followed by different letters in the same column are significant at the $p < 0.05$.
Abbreviations: ADG, average daily gain; DMI, dry matter intake; FCR, feed conversion ratio.

showed a reduction in ADG after 20 days consuming the plant. Interestingly, Wang et al. (2021) found an increase in ADG of sheep supplemented with the root of plants from the *Astragalus* genus. They attribute these results to the fact that the diet contained only the root of the plant, which could stimulate rumen microbes. Similarly, Abdalla et al. (2019) found that the animals supplemented with 10% and 15% *A. membranaceus* showed a moderate ADG (0.13 kg), compared to the control (0.12 kg). Additionally, the animals receiving the highest amount of AM also had the highest feed conversion ratio (Table 2).

Finally, the residual concentration of nitrocompounds in plasma after the supplementation with AM is shown in Figure 3a,b. The maximum concentration of 3NPA was found in the animals receiving the 0.5 g AM kg^{-1} BW dose ($p < 0.05$) in both sampling periods. The highest concentration of 3NPA in plasma observed in this study was 8 ppm, which could be consider non-toxic, as studied in rats in response to 2.5 and 3.75 mg kg^{-1} day^{-1} of 3NPA for male and female rats, respectively, with no observed adverse effect level. Moreover, concentrations of 25 μg kg^{-1} day^{-1} or 1.750 mg kg^{-1} day^{-1} for an ADI for a 70 kg human are considered safe for humans (Burdock et al. 2001), even considering that the chronic consumption even of small amounts of these compounds could be of risk for humans due the neurotoxicity of 3NPA, as was noted by Palfi et al. (1996). On the other hand, the concentration of 3NPA between periods was unaffected by treatment. Regarding the 3NPOH, even with the no toxicity per se of this nitrocompound, their quantification is necessary due to their enzymatic conversion into 3NPA in the liver (Anderson et al. 2005). In this regard, the concentration of 3NPOH was not different ($p > 0.05$) between treatments nor between periods.

These data allow us to assume that at this level of supplementation of AM, the rumen reaches the minimum amount of nitrocompounds to start the growth of *Denitrobacterium detoxificans*, the only known bacterium capable of metabolizing 3NPA (Anderson et al. 2000).

4 | Conclusions

The use of AM as a natural source for anti-methanogenic nitrocompounds can be an effective alternative to replace chemical synthetic compounds in animal feeding.

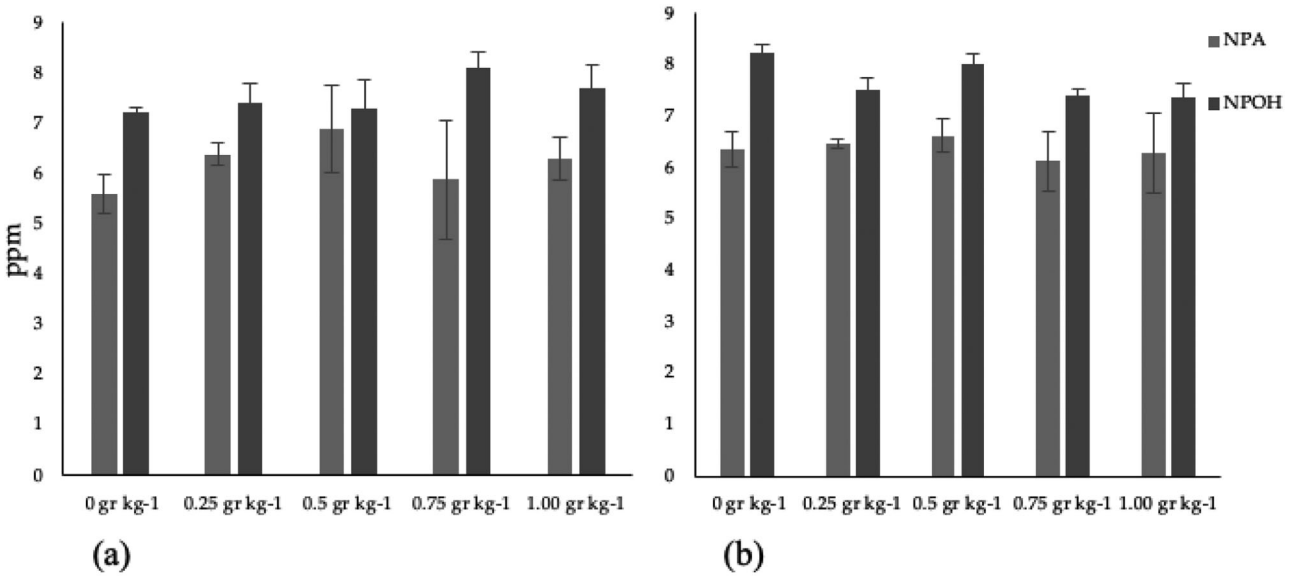

FIGURE 3 | Nitrocompound concentration in plasma on Days 30 (a) and 60 (b) of sheep supplemented with *Astragalus mollissimus*. Least squares mean (±standard error) of 3-nitro-1-propionic acid (3NPA) and 3-nitro-propanol (3NPOH) of *A. mollissimus* supplementation treatments.

According to the data, the use of AM as a natural strategy to mitigate GHG emissions is considered safe for animal-origin foods and, for instance, for humans.

According to the data observed for methane produced, the appropriate dosage could be between 0.25 and 0.75 g kg^{-1} BW day^{-1}, avoiding the risk of intoxication for animals.

Research regarding the agronomy or domestication of AM is needed.

Author Contributions

Agustin Corral-Luna, **Robin C. Anderson**, **Alberto Muro-Reyes**, **Martha M. Arevalos-Sánchez** and **Jagoba Rey-Gotxi**: conceptualization. **Jagoba Rey-Gotxi**, **L. Yuviana Martínez-Cordova**, **Martha M. Arevalos-Sánchez**, **Monserrath Felix-Portillo** and **America Chávez-Martínez**: methodology. **Jagoba Rey-Gotxi** and **L. Yuviana Martínez-Cordova**: validation. **Felipe A. Rodríguez-Almeida** and **Adrián O. Máynez-Pérez**: formal analysis. **Jagoba Rey-Gotxi**, **L. Yuviana Martínez-Cordova** and **Martha M. Arevalos-Sánchez**: investigation. **Agustin Corral-Luna** and **Jagoba Rey-Gotxi**: writing original draft preparation. **Jagoba Rey-Gotxi**, **Agustin Corral-Luna**, **Aser García**, **Robin C. Anderson**, **Einar Vargas Bello-Pérez**, **Alberto Muro-Reyes**, **Aleksandar K. Božić**, **Martha M. Arevalos-Sánchez**, **Monserrath Felix-Portillo** and **Adrián O. Máynez-Pérez**: writing review and editing. **Agustin Corral-Luna** and **Alberto Muro-Reyes**: supervision.

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Ethics Statement

Animals were maintained and cared for according to procedures approved by the University of Chihuahua animal care and use guidelines (Ethic Committee Name: Comité Institucional de Bioética; Approval Code: Decision No. P/302/2017, Approval Date: 2017) and the Mexican Government guidelines for the use of research animals (NOM-062-ZOO-1999). All experiment procedures were carried out in the facilities of the Facultad de Zootecnia y Ecología of the Universidad Autónoma de Chihuahua (Chihuahua, Mexico).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data presented in this study are under request of patent and are subject to intellectual property rights.

Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/vms3.70389.

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