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Supplementation of high-quality fresh forage to lambs fed a total mixed ration increased *in vitro* ruminal fermentation and digestibility

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The supplementation of fresh alfalfa into the diet of growing lambs fed with decreasing levels of a total mixed ration (TMR) was studied for its effect on *in vitro* ruminal fermentation activity. Twenty-four catheterized lambs [25.2 ± 3.67 kg body weight (BW)] were assigned to one of the following treatments: "TMR100"—TMR *ad libitum*; "TMR75'" and "TMR50"—TMR at 0.75 and 0.50 of potential intake, respectively, supplemented with alfalfa; and 'TMR0'—only alfalfa *ad libitum*. *In vitro* gas production kinetics and true digestibility (IVTD) were evaluated using the rumen liquid as inocula. Ruminal pH values and NH_3-N and volatile fatty acid concentrations were studied at the same time as inocula extraction. As the amount of alfalfa in the diet increased—by decreasing the level of TMR—*in vitro* gas production, ruminal pH values, NH_3-N concentrations, and acetic acid proportions linearly increased (p = 0.005, 0.008, 0.004, and 0.018, respectively). IVTD tended to linearly rise (p = 0.083) and the fermentation rate (p = 0.004) and propionic acid proportion (p< 0.001) linearly decreased. We conclude that the increase in the level of fresh alfalfa resulting from the decrease in TMR levels in lambs' diet positively impacted rumen fermentation activity and *in vitro* digestibility through the promotion of a suitable environment for ruminal microbiota.

KEYWORDS

alfalfa, growing lambs, ruminal fermentation, true digestibility, microbial activity

1 Introduction

Ruminant production systems in several parts of the world are based on temperate pastures, which usually have high levels of crude protein (CP) content and highly digestible content (Pearson et al., 1997; Pontes et al., 2007; Cajarville et al., 2015). These systems are facing various challenges regarding animal welfare and sustainability (Temple and

Manteca, 2020; Rivero and Lee, 2022). Even so, they offer opportunities and have several advantages over feedlot systems, in terms of expressions of natural animal behavior and final product quality (De Brito et al., 2018; Rivero and Lee, 2022), sustainability of the way of life of human communities, protection of environments, and use of food inedible for humans (Wilkinson and Lee, 2018; O' Mara et al., 2021; Wang et al., 2021). One key limiting factor of grazing systems is that pasture quality and availability are weather dependent and variable. This hinders the setting of production targets and is a limiting factor for animal productivity during some periods of the year.

On the other hand, the use of total mixed rations (TMRs) is a strategic tool in intensive production system feedlots. This kind of diet contains well-known nutrient levels, allowing for maximum animal production and efficiency throughout the year.

As an alternative, the combined use of both pasture and TMRs, alternated at different points in the day, has improved animal performance in dairy cows (Bargo et al., 2001; Mendoza et al., 2016; Santana et al., 2017). Although farmers have been using this feeding system to intensify production, the information available on this system is still scarce, particularly in sheep (Fernandez-Turren et al., 2020).

In another paper derived from the present experiment (Pérez-Ruchel et al., 2017), we reported that the decrease in the level of TMRs led to an increase of alfalfa dry matter (DM) and nutrient intake in lambs. However, contrary to expectations, these differences in intake were not related to the digestibility of diets measured *in vivo*, as the diets that contained more alfalfa were less digested than those that contained more TMRs, without differences in digesta transit.

According to Oba and Allen (1999), *in vitro* approaches would be more appropriate to evaluate some aspects of digestion because they reduce the interferences that could be generated by the *in vivo* measurements, such as rumen retention times or exposure to different gastrointestinal tract conditions. Therefore, through an *in vitro* evaluation of rumen digestion, we could explain the higher intake observed with decreasing TMRs and increasing alfalfa in the diet, which was not consistent with *in vivo* digestibility results (Pérez-Ruchel et al., 2017). We hypothesized that increasing the proportion of alfalfa in the diet would contribute to a more suitable rumen environment for fiber digestion, which could be ascertained through the evaluation of the rumen liquor activity of sheep consuming different proportions of alfalfa and TMRs. Therefore, this study aimed to evaluate the activity of the rumen environment of lambs consuming different proportions of alfalfa and TMRs, using *in vitro* methods for evaluation.

2 Materials and methods

The study was carried out at the Experimental Farm of the Facultad de Veterinaria, UdelaR, Uruguay (San José Department, GPS coordinates: latitude 34′40.652″ S; longitude 56′32.349″ W). All procedures involving animals were approved by the Bioethics Committee of the Facultad de Veterinaria (UdelaR, Uruguay, CNEA-CHEAUdelaR-CEUAFVet, d. 295/99).

2.1 Animals, diets, and experimental design

This experiment was performed using the same animals, diets, and experimental design published by Pérez-Ruchel et al. (2017). Briefly, 24 Corriedale × Milchschaf lambs (age 4 months), with an average body weight (BW) of 25.2 ± 3.67 kg, fitted with permanent rumen catheters, were individually housed in metabolism cages and fed with decreasing levels of TMRs and fresh alfalfa (*Medicago sativa*) *ad libitum*. The experiment was a randomized complete block design, with a 27-day experimental period (21 days of adaptation and 6 days of measurements). Lambs were divided into blocks according to BW (i.e., six blocks) and randomly assigned to receive one of the following treatments: TMR100—TMRs offered *ad libitum*; TMR75—TMRs at a level of 0.75 of the potential intake complemented with fresh alfalfa *ad libitum*; TMR50—TMRs at a level of 0.50 of the potential intake complemented with fresh alfalfa *ad libitum;* and TMR0—fresh alfalfa *ad libitum*.

The TMRs were formulated to meet growing lambs' requirements, with an estimated daily gain of 300 g according to the National Research Council (NRC, 2007) regulations (Table 1). For all treatments, TMRs were prepared daily and offered at 09:00 (hour 0), without restriction in quantity for animals receiving TMR100 treatment. The level of TMRs for the TMR75 and TMR50 treatments was fixed according to the potential intake and provided in one meal.

TABLE 1 Ingredients used and nutrient composition of total mixed rations (TMRs) and alfalfa [g/kg dry matter (DM), unless noted].

	TMR	Alfalfa
Ingredients of TMR		
Whole-plant corn silage (CS)	600	
Cracked corn grain	120	
Pelleted soybean meal	250	
Sodium bicarbonate (99% purity)	12	
Calcium carbonate (98.5% purity)	10	
Ammonium chloride (99.6% purity)	5	
Vitamin–mineral premix*	2	
Nutrient composition†		
DM, g/kg as-fed basis‡	401 (25.3)	296 (30.5)
OM, g/kg DM§	940 (2.1)	905 (0.2)
aNDFom, g/kg DM¶	354 (65.3)	374 (26.8)
ADFom, g/kg DM£	172 (3.9)	211 (3.1)
CP, g/kg DM#	198 (17.0)	209 (26.1)
NSC, g/kg DM&	363	305
Water-soluble carbohydrates, g/kg DM	65.2 (15.7)	96.8 (10.1)

*, mix of iron, copper, magnesium, manganese, calcium, phosphorus, zinc, sodium chloride, vitamins A, D_3, E, B_1, B_2, B_6, and B_{12}, nicotinamide, and calcium pantothenate; †, mean values (standard deviation) from samples taken from day 22 to day 31 (n = 10/feed); ‡, dry matter; §, organic matter; ¶, neutral detergent fiber assayed with a heat-stable amylase, sodium sulfite, and corrected for blank and ash-free content; £, acid detergent fiber corrected for blank and ash-free content; #, crude protein (CP); &, non-structural carbohydrates (NSC).

Potential intake was individually calculated by measuring voluntary intake during a period of 15 days. Each animal was supplied with 0.75 or 0.50 of their respective individual intake, according to the treatment.

The forage was collected from one paddock and was predominantly alfalfa, with the following botanical composition: 792 g/kg DM of alfalfa, 156 g/kg DM of *Lolium multiflorum*, 10 g/kg DM of *Lotus corniculatus*, and 42 g/kg DM of senescent forage and herbs. Alfalfa was cut daily at 13:00 with a disk mower (5 cm in height from the ground). The pre-cut biomass was 1,475 kg DM/ha (chemical composition in Table 1). Animals receiving the TMR0 treatment were offered an unrestricted quantity of fresh alfalfa, beginning at hour 5 after the morning feeding, and continuously throughout the day. Animals receiving treatments TMR75 and TMR50 received an unrestricted amount of fresh alfalfa after they finished the TMR ingestion. Drinking water was provided in buckets connected to running water, at room temperature (i.e., at ≈ 15° C), and was freely available.

The total DM intake of lambs was 0.75, 0.92, 0.89, and 1.08 kg/day for those receiving TMR100, TMR75, TMR50, and TMR0 treatments, respectively, and linearly increased as TMRs in the diet were reduced ($p < 0.001$). Alfalfa represented 0%, 44%, 62%, and 100% of the diet in TMR100, TMR75, TMR50, and TMR0, respectively (Pérez-Ruchel et al., 2017).

2.2 Measurements, sampling, and chemical analysis

The rumen activity was evaluated through *in vitro* gas production kinetics and *in vitro* true digestibility (IVTD). Rumen liquor was collected from each animal at hour 2 after the morning feed on day 22 of the experimental period.

The IVTD of the alfalfa used for the experiment (Table 1) was determined using DAISY equipment (DAISY® *in vitro* rumen fermenter, Ankom Technology Corp., Fairport, NY, USA) with 400 mL of rumen liquid for each treatment. Liquor was combined within each treatment and immediately used as inocula to evaluate IVTD. The alfalfa sample was ground to pass through a 2-mm screen and was incubated in porous bags for 48 hours (two replicates). After incubation, the bags were washed for 1 hour at 100°C in a neutral detergent solution and dried in an air-forced oven at 105°C to a constant weight. The IVTD was calculated as the percentage of material disappearing after incubation and washing procedures. The same procedure was performed two times (two batches, beginning on days 22 and 25).

For the evaluation of ruminal fermentation activity, the liquor of each animal was individually inoculated to measure gas production kinetics. Samples of whole-plant corn silage (180 g/kg DM, 344 g/kg ADFom, 81.1 g/kg CP, DM basis), corn grain (890 g/kg DM, 32 g/kg ADFom, 136 g/kg CP, DM basis), and alfalfa used for the experiment (Table 1) were used as substrates. These feedstuffs were ground to pass through a 1-mm screen, and weighed into 125-mL flasks (one flask/substrate/animal + two blanks/animal, total = 120 flasks), which were filled with 40.5 mL of a buffer solution (Williams et al., 2005) and 10 mL of strained rumen fluid, sealed with a rubber septum plus a crimp seal, and incubated for 96 hours in a water bath at 39°C, following the technique described by Mauricio et al. (1999). All incubation procedures were performed under a CO_2 stream. Internal pressure in flasks was recorded at 2, 4, 6, 8, 10, 12, 18, 24, 48, 72, and 96 hours of incubation with a D1005PS manometer (Ashcroft®, Stratford, CT, USA) and a hypodermic needle. After each reading the gas was vented. Pressure readings were converted into volume using the equation obtained in a previous experiment under similar conditions:

$$V = 4.40 \times P + 0.09 \times P^2$$

where V (mL) is the gas volume, and P (psi) is the observed pressure ($R^2 = 0.998$). The accumulated volume of gas for each incubation time until 96 hours was fitted to the model:

$$Gas = A\ \{1 - e^{[-kd(t-lag)]}\}$$

where A (mL) is the potential gas production, kd (h^{-1}) is the rate of gas production, and lag (h) is the lag time.

Moreover, ruminal fluid samples were collected from each animal using the permanent rumen catheters at hour 2 (day 22). Ruminal pH was immediately measured using a digital pH meter (eChem Instruments Pte., Oakton, Singapore). Two samples (1 mL) were mixed with 0.02 mL of sulfuric acid (50%, v/v) and two samples were mixed with 1 mL of perchloric acid (0.1 M) and frozen for later determination of NH_3-N and volatile fatty acid (VFA) concentrations, respectively.

The NH_3-N concentration in ruminal samples was analyzed by spectrophotometry according to Weatherburn (1967) using a spectrophotometer (BEL Photonics®, S-2000, SP, Brazil). The VFA concentrations were analyzed in accordance with Adams et al. (1984) using high-performance liquid chromatography (Dionex Ultimate® 3000, Waltham, MA, USA) with an Acclaim Rezex Organic Acid H+ (8%) and a 7.8 mm × 300 mm column at 210 nm. The total VFA concentration was calculated as acetate + propionate + butyrate concentrations (mM) and each VFA concentration was expressed as a percentage of the total VFA concentration.

Feed samples were analyzed for DM, OM, and N in accordance with the Association of Official Analytical Chemists (AOAC, 1990, methods ID 934.01, ID 942.05, and ID 984.13, respectively); neutral detergent fiber assayed with a heat-stable amylase, sodium sulfite, and corrected for blank and ash-free content (aNDFom) and acid detergent fiber corrected for blank and ash-free content (ADFom) were analyzed according to Robertson and Van Soest (1981), using a Tecnal fiber analyzer (TE-149, Tecnal, Piracicaba, SP, Brazil), expressed without residual ash and corrected for blanks, as recommended by Mertens (2003). The aNDFom was analyzed using sodium sulfite and amylase. Crude fat content (CF) was determined, according to Nielsen (2003), using a Goldfisch fat extractor (Goldfisch, Labconco 35001, TX, USA) under a petroleum ether reflux at 180°C for 3 hours; water-soluble carbohydrates (WSCs) (Yemm and Willis, 1954) were also analyzed. In addition, the non-structural carbohydrate (NSC) content of feeds was calculated in accordance with Sniffen et al. (1992) as:

$$100 - \left(aNDFom\ \left[\frac{g}{100}g\right] + CP\ \left[\frac{g}{100}g\right] + CF\ \left[\frac{g}{100}g\right] + ash\ \left[\frac{g}{100}g\right]\right)$$

2.3 Statistical analysis

Data were analyzed using the MIXED procedure in SAS (version 8.2; SAS Institute, Cary, NC, USA). Data related to gas production parameters were compared between treatments (inocula) as repeated measures over the flask, using the model:

$$Yijk = \mu + Ti + Sj + (TxS)ij + eijk,$$

where Y_{ijk} is the dependent variable, μ is the general mean, T_i is the fixed effect of the treatment, S_j is the fixed effect of the substrate, $(TxS)_{ij}$ is the interaction between inoculum and substrate, and e_{ijk} is the residual error.

The IVTD data were compared between treatments (inocula) as repeated measures over the batch, using the model:

$$Yij = \mu + Ti + eij,$$

where Y_{ij} is the dependent variable, μ is the general mean, T_i is the fixed effect of the treatment, and e_{ij} is the residual error.

The pH values and VFA and NH_3-N concentrations were analyzed according to the model:

$$Yijk = \mu + Ti + Bj + eijk,$$

where Y_{ijk} is the dependent variable, μ is the general mean, T_i is the fixed effect of the treatment in $_k$ animal replicates, B_j is the random effect of the block, and e_{ijk} is the residual error.

The effect of decreasing levels of TMRs in the diet (1.0, 0.75, 0.50, and 0.0) on the average values was evaluated by linear and quadratic regression.

Significant differences were declared if $p \leq 0.05$ and $0.05 < p < 0.10$ was considered to indicate a tendency toward significance.

3 Results

Total gas production (*A*) linearly increased ($p = 0.005$) and the fermentation rate (*kd*) linearly decreased ($p = 0.004$) as the levels of alfalfa increased and TMRs decreased (Table 2 and Figure 1). The three substrates incubated (corn silage, corn grain, and alfalfa) produced different gases, at different rates (effect of the substrate $p < 0.001$, not shown in the table), but with a similar response, with no interactions between treatment and substrates ($p > 0.05$, not shown in the table).

The IVTD tended to rise linearly ($p = 0.083$) as the levels of TMRs decreased and alfalfa increased (Table 2).

Ruminal pH values increased linearly and quadratically ($p = 0.08$ and 0.041, respectively) at decreasing rates with the increase of alfalfa in the diet. NH_3-N concentrations also increased linearly and quadratically ($p = 0.004$ and 0.013, respectively), but at increasing rates. Meanwhile, the total VFA concentrations presented no differences between treatments, but some percentages of individual fatty acids varied. The concentration of acetate linearly increased (from 46.6% to 50.2% of total VFAs, $p = 0.018$), and propionate linearly decreased (from 34.7% to 30.6% of total VFAs, $p < 0.001$) with the reduction of TMRs and increase of alfalfa (Table 2).

4 Discussion

The higher total gas production and IVTD of lambs with diets with higher levels of alfalfa and lower levels of TMRs indicate an improvement of rumen activity, which *a priori* can be considered contrary to expectations, since the NSC concentration was 20% higher in TMRs than in alfalfa. In fact, Chen et al. (2022), working *in vitro* with different proportions of rumen-degradable starch and

TABLE 2 *In vitro* gas production parameters and true digestibility (IVTD) using ruminal liquor of lambs fed total mixed rations (TMRs) (TMR100), 75% TMR and alfalfa (TMR75), 50% TMR and alfalfa (TMR50), or only alfalfa (TMR0) as inocula, and ruminal liquor conditions at the same hour of the inocula extraction (pH values and NH_3-N and VFA concentrations).

	TMR100	TMR75	TMR50	TMR0	SEM*	$p^{\dagger}$		
						T	L	Q
In vitro ruminal fermentation activity								
A‡	164	200	207	231	9.62	<0.001	0.005	0.420
kd§	0.120	0.105	0.095	0.072	0.01	<0.001	0.004	0.910
lag¶	1.34	1.01	1.44	1.44	0.17	0.146	0.407	0.668
IVTD (%)	80.8	78.7	83.5	84.9	1.039	<0.001	0.083	0.584
Ruminal conditions								
pH	5.91	6.24	6.41	6.42	0.099	0.010	0.008	0.041
NH_3-N (mg/dL)	21.1	19.9	18.7	31.8	2.190	0.002	0.004	0.013
Total VFA (mM)	194	185	209	199	14.80	0.741	0.636	0.786
Acetate (%)	46.6	46.0	47.5	50.2	1.133	0.087	0.018	0.424
Propionate (%)	34.7	34.3	32.8	30.6	0.689	0.004	<0.001	0.692
Butyrate (%)	18.7	19.7	19.6	19.2	0.927	0.905	0.941	0.489

*, standard error of means; †, significance level, effect of treatment (T) and linear (L) and quadratic (Q) effect of decreasing level of TMR in the diet; ‡, gas production (mL/DM incubated, g); §, rate of gas production (h^{-1}); ¶, lag time (h).

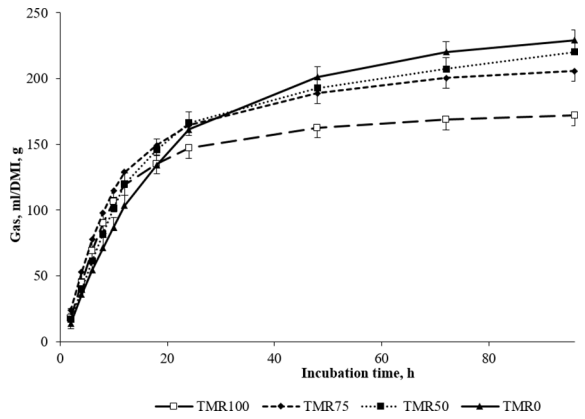

FIGURE 1
In vitro gas production (mL/DM incubated, g) using ruminal liquor of lambs fed TMR (TMR100), 75% TMR and alfalfa (TMR75), 50% TMR and alfalfa (TMR50), or only alfalfa (TMR0) as inocula (mean values ± standard error).

rumen-degradable protein, observed that gas production increased with the increase in starch provided by degradable grains.

It is interesting to note that the type of fermentation promoted by the alfalfa forage (see TMR0 in Figure 1) was slower but more extended, with greater total fermentation than the other combinations. This type of fermentation would lead to a suitable environment for ruminal microbiota, especially for fibrinolytic communities, which are sensitive to ruminal variations and drops in pH (Zhang et al., 2017) and have crucial importance in ensuring fiber digestion of feeds (Firkins, 2021). The fact that the *in vitro* digestibility was measured for a relatively long time (48 hours) explains the strong tendency of higher IVTD observed in animals consuming more alfalfa.

According to Dryhurst and Wood (1998), when available N is not limiting, as in this case, gas production depends on carbohydrate degradability. In this sense, TMRs and alfalfa had not only different NSC concentrations but also different NSC partitioning. In TMRs the WSC fraction represented 18% of the total NSC concentration, whereas in alfalfa WSC represented 32% of the total NSC concentrations. The positive action of WSC on microbiota activity is well known (Russell, 1993) and can be associated with the increase of *in vitro* digestibility and gas production, as reported by Cajarville et al. (2015).

The higher pH values, NH_3-N concentrations, and acetate percentages and the lower propionate levels observed as alfalfa increased in the diet are explained by the lower fiber and CP content of TMR. The greater WSC proportion in the forage compared with TMRs was not reflected in a higher butyrate concentration as observed by Heldt et al. (1999). Although this measurement corresponds to only one sample extracted simultaneously with the liquid extraction for *in vitro* assays, the results are consistent, in general terms, with data of ruminal environment observed by Pérez-Ruchel et al. (2017) throughout 24 hours on another day of the same experiment. In the mentioned paper, the average pH values and NH_3-N concentrations throughout the day linearly increased as TMRs were reduced and alfalfa was increased in the diets. The quadratic effect observed for these variables in this study was probably related to the time of measurement, which was 2 hours after TMR provision in treatments T0, T50, and T75, and, therefore, with NSC entering the rumen.

Overall, these results can explain the higher DM and OM intake observed *in vivo* as TMRs were reduced and alfalfa increased in the diets (Pérez-Ruchel et al., 2017), and indicate that digestible fibers and sugars, instead of other NSCs (e.g., starch), promote a sustained fermentation that would favor intake. It can also be hypothesized that the higher intake levels of the treatments with high levels of alfalfa observed by Pérez-Ruchel et al. (2017) were due to a preference of the animals for alfalfa, as observed in dairy cows (Buse et al., 2022), and this higher intake level led to a more active ruminal environment, as observed in this study, owing to higher rates of substrate entry and content turnover (Mitsumori et al., 2019).

Whatever the causality, this study shows the positive link between the ingestion of high-quality, fresh forage and the rumen fermentation activity of lambs.

5 Conclusion

Increasing levels of high-quality, fresh alfalfa, by decreasing levels of TMRs in the diet of lambs positively impacted rumen fermentation activity and *in vitro* digestibility. This result could be associated with the high-quality fiber and WSC contents of the fresh forage, which promoted a suitable environment for ruminal microbiota and fermentation activity.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Comisión de Ética en uso de Animales (CEUA, Committee on Ethics in the Use of Animals).

Author contributions

AP-R participated in the design of the work, data acquisition (laboratory analysis), analysis and interpretation of results, and writing of the manuscript. JR participated in the design of the work, interpretation of results, and writing of the manuscript. CC participated in the design of the work, interpretation of results, and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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