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Moringa Extract to Modulate Rumen Fermentation and Lactation Performance of Ewes

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Abstract

This study aimed to evaluate the supplementation of aqueous extract of Moringa oleifera (AEMO) as a natural ruminal modulator to improve the lactation performance of ewes. The AEMO was prepared by chopping *Moringa oleifera* leaves and diluting them in distilled water (163.3 g DM/L). Twelve ewes were used in a replicated 3 × 3 Latin square, with periods of 14 days (assessments on the last five days of each period). Treatments were as follows: 20 mL of water as Control, 20 mL of AEMO (20-AEMO), and 40 mL of AEMO (40-AEMO). Ewes were milked twice a day (7:30 a.m. and 2:30 p.m.). Diet corresponds to grain mix (at 3% of BW) and hay ad libitum. We determined the intake, digestibility, fermentative measurements, metabolic measurements, and milk production and composition. Intake and digestibility were not affected by AEMO. Milk yield and the concentrations of fat, protein, and lactose were numerically lower in ewes supplemented with 20-AEMO. A linear decrease in milk protein yield was observed when the highest extract level (40-AEMO) was used. Ruminal pH did not differ among treatments; however, there was a tendency for reduced acetate and increased propionate concentrations, which corresponded with a non-significant numerical decrease in methane estimates in 40-AEMO group. Blood and urinary parameters were not affected by AEMO supplementation. Inclusion of Moringa extracts as an additive in lactating ewes diet does not affect intake and nutrient digestibility, but tends to affect ruminal fermentation and microbial synthesis, with possible changes in methane emission estimation, and impair milk protein production. Therefore, we recommend studies with different extract concentrations to investigate possible effects on rumen fermentation and the synthesis of milk compounds.

Keywords: digestibility; fermentation; methane; natural additives

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1. Introduction

Ionophores act on the rumen microorganism population, favoring its proper functioning [1]. This chemical additive is commonly used in animal production and affects

Gram-positive bacteria, allowing most of the substrate to be used by Gram-negative bacteria [2], which results in higher propionic acid production, increasing the energy supply for the animal [3]. The ionophore is used with some positive points in animal production; however, its continuous use in the animal diet can result in possible accumulated residues eliminated via excreta in the environment [4], which should be taken into consideration.

To increase sustainability in ruminant production, natural additives are an excellent alternative to replace antibiotics and ionophores. Plants rich in fatty acids [5], vitamins [6], and bioactive compounds with antimicrobial properties [7] have the potential to be used for natural additive production. Moringa oleifera and its extracts have these characteristics; thus, it has been studied as an option to improve animal performance and efficient use of dietary nutrients [8,9]. All parts of Moringa are edible and can be used in several ways [10].

Studies have reported the benefits of using different parts of Moringa on ruminant performance [9,11,12]. Extracts from Moringa leaves were evaluated through in vitro assays and characterized as promising to increase the quality of ruminant products and health-related characteristics [10]. The use of aqueous extract of fresh Moringa leaves in in vitro fermentation indicates a potential increase in volatile fatty acids without changes in fatty acid proportions [13]. Aqueous extract of *Moringa oleifera* (AEMO) (125 g of DM/L) improved animal performance of lactating goats receiving 10, 20, or 30 mL of AEMO, along with increased digestibility [9]. Oral administration of AEMO in sheep had beneficial effects against *Fasciola gigantica* and *Clostridium novyi*, which are common parasites and bacteria found in the gastrointestinal tract that cause infection [14].

However, based on our knowledge, few studies have reported the effect of AEMO on lactating ewe performance. Thus, we sought to evaluate the effect of AEMO as an additive on rumen fermentation, metabolic measurements, and performance of lactating ewes. We hypothesize that AEMO compounds will act on ruminal microorganisms, altering the fermentation pathways, hence improving the lactating ewe performance.

2. Materials and Methods

The experiment was carried out at the Federal University of Grande Dourados, located in Dourados-MS, Brazil (22°11′55″ S, 54°56′7″ W, and 452 m altitude), with a tropical climate (wet summers and dry winters). During the trial, the average temperature was 26.4 °C, with a maximum of 30.3 °C and a minimum of 22.7 °C, according to the Embrapa Agropecuaria Oeste meteorological station. Animal management and sampling methodology were the same as those described previously by Chagas [15].

2.1. Aqueous Extracts of Moringa oleifera

Fresh *Moringa oleifera* leaves were harvested and stored at −20 °C until extract preparation. Leaves were chopped (approximately 1 cm) and diluted in distilled water based on the DM ratio (163.3 g of DM/L). The extract was heated at 30 °C for 24 h, followed by solid particle removal [13]. The aqueous extract was frozen in daily portions and thawed at 4 °C overnight before use.

The extracts were submitted to phytochemical prospecting [16] to confirm the classes of secondary metabolites [17]. We confirmed the presence of triterpenes and steroids via hydrolysis of the dry methanolic extract. This procedure was performed with potassium hydroxide (0.5 mol/L) and submitted to reflux for 1 h. Then, the compounds were extracted with ethyl ether and subjected to the Liebermann–Burchard reaction. To determine the presence of secondary metabolite classes, we assessed the intensity reactions [18], which are classified as follows: negative reaction (− = 0%), low intensity (+ = 10%), medium intensity (++ = 50%), and high intensity (+++ = 100%). The extracts were solubilized at 1 mg/mL in

methanol to analyze phenolic compounds, flavonoids, and tannins, and all analyses were performed in triplicate.

The content of phenolic compounds was determined based on the Folin–Ciocalteu colorimetric method [19]. For this, the stored solution of each sample (100 µL) was mixed with the other reagents and incubated in a dark environment at 23 ± 2 °C for 2 h. Their absorbances were recorded at a wavelength of 760 nm. The results were expressed as mg of gallic acid per L of extract (mg GAE L-1). The flavonoid content was determined according to the methodology described by Djeridane [19]. For this, the stored solution of each sample (1000 µL) was mixed with the other reagents and incubated in a dark environment at 23 ± 2 °C for 15 min. Their absorbances were recorded at a wavelength of 430 nm. The results were expressed as mg of flavonoids rutin per L of extract (mg RUE L-1).

The tannin content was determined using the Folin–Denis spectrophotometric method [20]. The sample (2 mL) was mixed with 2 mL of Folin–Denis reagent and 2 mL of 8% sodium carbonate. After reacting for 2 h, the mixture was read in a spectrophotometer at a wavelength of 725 nm, using water as a blank sample. The results were expressed as mg of tannic acid equivalent per L of extract (mg TAE L-1).

The extract's antioxidant activity was analyzed by measuring the radical scavenging activities of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP), flavonoid and polyphenol contents, and lipid oxidation (thiobarbituric acid-reactive substances, TBARS). The ABTS radical scavenging activity (%) is a measure of the extract's ability to scavenge the 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical, determined by colorimetry according to Re [21]. DPPH radical scavenging activity (%) was quantified by colorimetry based on antioxidant-mediated electron transfer, which reduces DPPH (purple color) to diphenyl-picrylhydrazine (yellow color) according to Bondet [22]. FRAP was determined according to Zhu [22]. Polyphenols were determined with Folin–Ciocalteu reagent and gallic acid as a reference standard, according to Singleton and Rossi [23]. Extract lipid oxidation was based on TBARS, determined according to Souza [24] methodology.

2.2. Animals and Experimental Design

Twelve Pantaneira ewes with 87 ± 4.33 days in milk (DIM) and 46.7 ± 4.19 kg BW were used. The ewes were placed in individual pens (2 m^2) with rice husk bedding. The ewes were weighed and had their body condition evaluated every 14 days. The ewes were milked at 07:30 a.m. and 14:30 p.m. Ewes were fed at 8:00 a.m. on individual feeders, and the amount offered and orts were weighed daily to determine the intake. Oat hay (*Avena sativa*) and water were provided ad libitum (considering 10% of orts). The grain mix was supplied at the rate of 3% of BW and was formulated with soybean meal, ground whole corn, wheat meal, urea, limestone, manganese sulfate, zinc sulfate, iron sulfate, potassium iodate, sodium selenite, Vitamin A, Vitamin D3, Vitamin E, Cobalt Sulfate, and Q.S.P to meet ewe requirements. The diet's chemical composition is shown in Table 1. The diet was provided in two portions, 60% after morning milking and 40% after afternoon milking. Ewes received the same diet during the entire experimental period, only varying the concentration of supplemented extract.

The experiment was conducted in a 3 × 3 Latin square design and replicated four times, with three treatments, three periods of 14 days (9 days for adaptation and 5 days for data collection, allowing sufficient washout between treatments), and 12 ewes. Ewes were blocked and assigned to each Latin square based on milk production and body weight and then randomly assigned to each treatment in the Latin squares. Treatment sequences were randomized within each square to ensure balanced exposure and minimize potential sequence and carryover effects. Daily treatments used were a placebo with 20 mL of water

as Control, 20 mL of aqueous extract of *Moringa oleifera* (20-AEMO), and 40 mL of aqueous extract of *Moringa oleifera* (40-AEMO). Treatments were administered orally every day after morning milking with a dosing gun to ensure they received the entire portion.

Table 1. Chemical composition of grain mix and oat hay provided according to the 3% BW of lactating ewes.

Nutrients	Grain Mix	Oat Hay
Dry Matter, %	85.7	84.6
Organic Matter, %	95.4	93.2
Crude Protein, %	15.6	8.7
Ether Extract, %	5.5	0.6
Neutral Detergent Fiber, %	14.7	56.8
Acid Detergent Fiber, %	3.7	29.4
Non-Fiber Carbohydrate, %	59.3	26.1

2.3. Evaluation of Intake and Digestibility

Feed intake was obtained daily by the difference between what was offered and the leftovers. Leftovers were weighed before the morning meal. Diet ingredients and leftovers were sampled on the 11th, 12th, and 13th d of each period. We performed fecal spot sampling at 6 h and 12 h of d-11, at 8 and 14 h of d-12, and at 10 and 16 h of d-13, composited by animal and period. The samples were stored at (−18 °C) until analysis.

All samples were pre-dried at 65 °C in a forced-ventilation oven for 48 h and then weighed to obtain the pre-dried weight. The samples were ground using a Wiley mill with a 1 mm screen (Ramsey, MN, USA). Then, they were analyzed for dry matter (DM; ID 934.01), ash (ID 930.05), crude protein (CP; ID 981.10), and ether extract (EE, ID 920.39) contents according to AOAC [25]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined according to Van Soest [26]. Non-fiber carbohydrate (NFC) content was determined according to Detmann and Valadares Filho [27]. Organic matter (OM) content was obtained by the difference between dry matter and ash.

For digestibility assessment, we used indigestible NDF (iNDF) from fecal, leftovers, and feed samples as an internal marker, and it was assessed according to Huhtanen [28]. To determine iNDF, we weighed 25 mg/cm^2 of feed, orts, and feces in filter bags measuring 5 × 5 cm. We incubated the samples in duplicate for 288 h in cattle with rumen cannulas. Then, the bags were removed and washed in running water until they were completely clean and submitted to fiber analysis in neutral detergent [26].

2.4. Milk Production

Milk yield was recorded during the last five days of each experimental period. On days 10, 11, and 12, a composite milk sample (50 mL) was obtained daily by pooling equal volumes from the morning and evening milkings. Samples were stored at −20 °C until analysis. Milk fat, protein, casein, and urea nitrogen contents were determined according to Silva [29], lactose concentration according to Teles [30], and total solids following ISO [31] procedures. The content of non-fat solids was calculated as the difference between total solids and fat content.

2.5. Ruminal and Metabolic Parameters

Ruminal fluid was collected on the 14th day of each experimental period using an esophageal tube 3 h after feeding. The initial portion of the sample (approximately 50 mL) was discarded to avoid saliva contamination. Immediately after each collection, the ruminal fluid pH was determined with a digital potentiometer (Mpa-210, MS Tecnopon instrumentação, Piracicaba, SP, Brazil) calibrated with pH 7.0 and 4.0 buffers. A subsample was stored

at −80 °C for further VFA analysis. Volatile fatty acid analysis was performed as described by Santos [32]; in short, samples were centrifuged at 7000 rpm for 5 min and filtered through a nylon filter (0.22 µm). The analysis was performed with HPLC (Shimadzu, Prominence model, Kyoto, Japan). The HPLC was equipped with a model SPD-20 ultraviolet detector programmed to operate at a wavelength of 210 nm. In addition, the system was equipped with an Aminex HPX-87H column (Hercules, CA, USA) with dimensions of 300 × 7.8 nm and a particle diameter of 9 µm at 30 °C. The injection volume was 20 µL. The mobile phase consisted of 5 mM H_2SO_4 in the isocratic mode for 37 min. Methane was estimated using the equation [CH_4 = 0.45 acetate − 0.275 propionic + 0.40 butyrate] reported by Moss [33] based on the VFA profile.

Blood was sampled on the 13th day of each experimental period, 4 h after feeding. Vacutainer tubes with potassium fluoride were used for urea nitrogen analysis and heparin for glucose analysis. The tubes were centrifuged at 4000 rpm for 15 min, and then the plasma was stored at −18 °C until analysis. Glucose and urea were quantified by the colorimetric method (Gold Analyze Test, reaction time: 10 min at 37 °C).

Spot urine sampling was performed at the same time as feces sampling. It was diluted (1:5) in sulfuric acid solution (50%) and stored (−18 °C) to determine allantoin, creatinine, uric acid, and xanthine + hypoxanthine. A separate sample without acidification was stored (−18 °C) for urea determination. Creatinine was quantified using the colorimetric method (Gold Analyze Test, reaction time: 90 s at 37 °C, standard: 1 × 5 mL). Creatinine data were used to estimate urine excretion [34]. Urea was quantified using the commercial kit (Gold Analyze Test, reaction time: 10 min at 37 °C). Allantoin was quantified according to the method described by Young and Conway [35], as cited by Chen and Gomes [36]. Xanthine + hypoxanthine was determined by the method described by Chen and Gomes [36], with minor changes, which consists of evaluating the uric acid by a calorimetry kit (Teste Vida Biotecnologia, Belo Horizonte, MG, Brazil).

2.6. Statistical Analysis

Means were calculated for all measurements of each variable during the sampling period for each ewe. The resulting mean data were analyzed using the mixed procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA) according to a replicated 3 × 3 Latin square design. The model includes square, period, and treatment as fixed effects and the ewe within the square as a random effect.

$$Y_{ijkl} = \mu + S_i + P_j + T_k + A_{l(i)} + e_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, S_i = fixed effect of ith square, P_j = fixed effect of jth period, T_k = fixed effect of kth treatment, $A_{l(i)}$ = random effect of lth animal within ith square, and e_{ijkl} = is the residual error term.

Treatment allocation within each period was randomized to control for potential sequence and carryover effects. The Latin square structure inherently balanced treatment sequences across animals and periods, minimizing residual influences from previous treatments. Carryover effects were statistically evaluated by including treatment sequence and treatment × period interactions in the model. No significant carryover or residual effects were detected ($p > 0.10$), confirming that treatment responses were independent across periods. Model assumptions were evaluated by inspecting residual plots and testing for normality (Shapiro–Wilk) and homogeneity of variances (Levene's test). When necessary, data were log-transformed to meet these assumptions; however, untransformed means are presented for clarity. Responses to AEMO dose were evaluated using orthogonal polynomial contrasts (linear and quadratic). Significance was declared at $p \leq 0.05$, and tendencies were discussed at $0.05 < p \leq 0.10$.

3. Results

The phenolic compounds, flavonoids, and tannins had medium intensity (Table 2). Coumarins, triterpenes, steroids, cyanogenic heterosides, cardioactive heterosides, reducing sugars, and saponins had low intensity. On the other hand, the alkaloids had a negative reaction to the characterization intensity. The contents of phenolic compounds, flavonoids, and tannins in AEMO were 2417 mg/L, 1547 mg/L, and 196 mg/L, respectively. The AEMO lipid oxidation content (TBARS) was 17.2 mmol/kg of fat. The antioxidant activity of the aqueous extract was evaluated using the ABTS, DPPH, total polyphenols, and FRAP assays, yielding values of 230 µM ET, 39%, 73 mg GAE/L, and 109 mg GAE/L, respectively.

Table 2. Bioactive compounds and antioxidants from aqueous extracts of Moringa oleifera provided as a natural additive for lactating ewes.

Bioactive Compounds	Aqueous Extracts of *Moringa oleifera* [1]
Phenolic compounds	++
Flavonoids	++
Tannins	++
Coumarins	+
Triterpenes and steroids	+
Cyanogenic heterosides	+
Cardioactive heterosides	+
Reducing sugars	+
Saponins	+
Alkaloids	−
Phenolic compound content (mg/L)	2417
Flavonoids (mg/L)	1547
Tannins (mg/L)	196
Antioxidants	
ABTS (ET µM)	230
DPPH (%)	39
Polyphenols (mg EAG/L)	73
FRAP (mg EAG/L)	109
Oxidation	
TBARS (mmol/kg of fat)	17.2

[1] The presence of secondary metabolic compounds was classified as follows: negative reaction (− = 0%), low intensity (+ = 10%), medium intensity (++ = 50%), and high intensity (+++ = 100%).

3.1. Intake and Digestibility

Dry matter and organic matter intake of lactating ewes were not affected by AEMO supplementation, regardless of the dose (20-AEMO or 40-AEMO), with overall mean values of 1530 and 1441 g/d for DM and OM intake, respectively. The same was observed for CP, EE, NDF, and NFC, which were not affected by AEMO supplementation (Table 3), with averages of 260 g/d, 48 g/d, 301 g/d, and 826 g/d, respectively.

Supplementation with AEMO did not affect the digestibility parameters of lactating ewes (Table 3). The overall apparent digestibility coefficients of DM, OM, CP, EE, NDF, NFC, and total digestible nutrients averaged 57.1%, 61.7%, 54.6%, 82.8%, 36.1%, 77.5%, and 62.8%, respectively.

Table 3. Dry matter and nutrient intake and digestibility of lactating ewes receiving 10 mL/d of water (Control), 20 mL/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 mL/d of aqueous extract of *Moringa oleifera* (40-AEMO).

	Treatments [1]			SEM [2]	*p*-Value		
	Control	20-AEMO	40-AEMO		Treat	L	Q
C/F ratio [3]	80:20	81:19	82:18	67.35	0.49	0.24	0.88
Intake							
DM, g/d	1554	1515	1522	62.5	0.67	0.60	0.47
OM, g/d	1468	1417	1438	55.8	0.65	0.59	0.47
CP, g/d	264	256	263	9.3	0.66	0.88	0.37
EE, g/d	49	48	49	1.4	0.57	0.72	0.33
NDF, g/d	319	297	290	29.3	0.57	0.32	0.78
NFC, g/d	830	822	828	19.1	0.95	0.95	0.76
Digestibility							
DM, %	55.8	56.0	59.7	5.67	0.63	0.45	0.70
OM, %	60.6	60.4	64.3	5.16	0.58	0.43	0.63
CP, %	52.5	52.6	58.9	6.90	0.43	0.31	0.59
EE, %	81.5	83.5	83.6	4.96	0.88	0.64	0.82
NDF, %	33.4	37.8	37.1	6.41	0.88	0.70	0.52
NFC, %	74.0	75.6	83.0	5.68	0.41	0.20	0.73
TDN, %	61.7	61.6	65.2	4.95	0.60	0.44	0.66

[1] Treatments: 20-AEMO: 20 mL of aqueous extract of Moringa oleifera; 40-AEMO: 40 mL of aqueous extract of Moringa oleifera. [2] SEM: Standard error of the mean. [3] C/F ratio: Concentrate–Forage Ratio.

3.2. Milk Production

Milk production in g/day presented a quadratic behavior ($p = 0.04$), with the supply of 20 mL of extract, decreasing the production in 59.2 g/d, in relation to the Control, remaining similar between 40-AEMO and Control (Table 4). The milk protein content showed a linear behavior between the treatments, with a reduction in this content between treatments with AEMO. The other milk measurements, such as fat, CN, TS, defatted solids, and milk urea nitrogen (MUN), were not influenced ($p > 0.05$) by AEMO supplementation for lactating ewes, presenting averages of 27 g/d, 14 g/d, 101 g/d, 76 g/d, and 17 g/d, respectively (Table 4).

Table 4. Corrected milk production and milk composition of ewes receiving 10 mL/d of water (Control), 20 mL/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 mL/d of aqueous extract of *Moringa oleifera* (40-AEMO).

	Treatments [1]			SEM [2]	*p*-Value		
	Control	20-AEMO	40-AEMO		Treat	L	Q
Milk production, g/d	625	566	617	17.8	0.12	0.79	0.04
Fat, g/d	26.3	25.9	31.0	1.75	0.55	0.30	0.74
Protein, g/d	40.4	36.2	36.0	1.49	<0.01	<0.01	0.42
Casein, g/d	15.5	14.7	14.7	0.60	0.14	0.26	0.09
Lactose, g/d	23.9	21.2	23.7	0.84	0.84	0.80	0.59
Total Solids, g/d	104.6	99.0	102.3	4.08	0.20	0.87	0.08
Defatted solids, g/d	80.0	74.3	74.6	4.10	0.16	0.26	0.13
MUN, mg/dL	18.5	16.6	18.0	1.11	0.54	0.94	0.28

[1] Treatments: 20-AEMO: 20 mL of aqueous extract of Moringa oleifera; 40-AEMO: 40 mL of aqueous extract of Moringa oleifera. [2] SEM: Standard error of the mean.

3.3. Ruminal and Metabolic Parameters

AEMO supplementation did not influence ruminal pH, maintaining an average of 5.67. Similarly, there was no effect of AEMO on total VFA production (Table 5), with a mean of 108 mmol/L. The molar proportion of acetic acid, propionic acid, butyric acid, and valeric acid did not change with the inclusion of AEMO, maintaining the averages of 50.7%, 37.7%, 7.8%, and 1.0%, respectively. Consequently, the acetate–propionate ratio was not influenced by any AEMO level (Table 5), with an average of 1.4. Iso-valeric acid presented quadratic behavior ($p = 0.03$), increasing by 1.1 percentage units, in relation to the Control, when the ewes received 20 mL of AEMO, and decreasing to values similar to the Control when they were supplemented with 40 mL. No differences were observed among treatments for methane emission (Table 5), maintaining the average emission of 17.2 mmol/L and a ratio of 15.9 between methane and VFA.

Table 5. Ruminal measurements of lactating ewes receiving 10 mL/d of water (Control), 20 mL/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 mL/d of aqueous extract of *Moringa oleifera* (40-AEMO).

	Treatments [1]			SEM [2]	*p*-Value		
	Control	20-AEMO	40-AEMO		Treat	L	Q
pH	5.7	5.7	5.6	0.16	0.82	0.75	0.60
Total VFA, mmol/L	111.1	105.9	108.6	10.85	0.87	0.81	0.67
Acetic acid, %	54.3	50.2	47.7	3.42	0.15	0.06	0.78
Propionic acid, %	36.2	35.3	41.9	3.74	0.20	0.14	0.25
Butyric acid, %	7.8	8.4	7.2	2.45	0.88	0.80	0.65
Iso-valeric acid, %	1.2	2.3	0.9	0.51	0.03	0.55	0.01
Valeric acid, %	0.6	1.6	0.8	0.69	0.24	0.79	0.11
Acetate–propionate ratio	1.7	1.4	1.1	0.34	0.22	0.08	0.83
Methane, mmol/L	19.1	18.1	14.5	3.08	0.30	0.14	0.61
Methane/VFA ratio	17.5	17.0	13.4	2.59	0.25	0.12	0.47

[1] Treatments: 20-AEMO: 20 mL of aqueous extract of Moringa oleifera; 40-AEMO: 40 mL of aqueous extract of Moringa oleifera. [2] SEM: Standard error of the mean.

Blood glucose and urea levels were not influenced ($p > 0.05$) by AEMO supplementation for lactating ewes. Even so, numerically, 40-AEMO presented the lowest urea concentration (−7.2 mg/dL, Table 6). Furthermore, the urinary measurements evaluated urea, allantoin, uric acid, and xanthine plus hypoxanthine were not influenced by the addition of AEMO (Table 6), maintaining averages of 12.4 g/d, 10.9 mmol/d, 2.3 mmol/d, and 0.16 mmol/d, respectively. Allantoin means were numerically lower with AEMO, but the effect was not significant ($p = 0.44$).

Table 6. Glucose and urea concentration in blood, and urinary measurements of lactating ewes receiving 10 mL/d of water (Control), 20 mL/d of aqueous extract of *Moringa oleifera* (20-AEMO), or 40 mL/d of aqueous extract of *Moringa oleifera* (40-AEMO).

	Treatments [1]			SEM [2]	*p*-Value		
	Control	20-AEMO	40-AEMO		Treat	L	Q
Blood							
Glucose, mg/dL	67.3	65.5	66.0	2.17	0.68	0.55	0.54
Ureic nitrogen, mg/dL	66.5	64.0	59.3	4.50	0.29	0.12	0.78
Urine							
Urea, g/d	11.9	11.9	13.6	1.23	0.31	0.19	0.43
Allantoin, mmol/d	16.4	7.5	8.8	7.11	0.44	0.37	0.37

Table 6. *Cont.*

	Treatments [1]			SEM [2]	*p*-Value		
	Control	20-AEMO	40-AEMO		Treat	L	Q
Ureic acid, mmol/d	2.4	2.2	2.4	0.43	0.88	0.77	0.71
X + H [3], mmol/d	0.2	0.2	0.1	0.14	0.73	0.57	0.59

[1] Treatments: 20-AEMO: 20 mL of aqueous extract of Moringa oleifera; 40-AEMO: 40 mL of aqueous extract of Moringa oleifera. [2] SEM: Standard error of the mean. [3] X + H: Xanthine + Hypoxanthine.

4. Discussion

The use of an aqueous extract was intended to isolate and deliver the soluble bioactive compounds present in *Moringa oleifera* leaves, primarily phenolic acids, flavonoids, and other antioxidant metabolites, without introducing the fibrous plant matrix that could confound nutrient composition and digestibility. The extract allows a more standardized and concentrated delivery of the potentially active constituents while minimizing dietary dilution effects. The DM and nutrient intake observed in our study are evidence that the compounds of Moringa oleifera did not influence or affect the intake. However, Kholif [9], using AEMO with a lower concentration (125 g of DM/L), observed an increase in DM intake with an increase in AEMO supplied. Therefore, the possible effects of AEMO on intake may have different intensities depending on the variations in its phytogenic compound concentrations, depending on different leaf maturity at harvest or the crop location [37].

We hypothesized that ewes would improve ruminal fermentation efficiency and thus would better use the feed provided. Therefore, an increase in digestibility was expected, since Moringa bioactive compounds increase digestibility due to improved ruminal fermentation [38]. The lack of an effect on DM digestibility in our trial could be due to an overdose of AEMO (163.3 g of DM/L). Since Kholif [9] observed an increase in DM digestibility when AEMO at a lower concentration (125 g of DM/L) was administered to lactating goats.

The numerical differences observed in the VFA proportions indicate a change in rumen fermentation. Moreover, we observe a numerical reduction in acetic acid ($p = 0.15$) for treatments with AEMO and a numerical increase in propionic acid ($p = 0.20$) in the 40-AEMO treatment. This balance between the proportion of propionic acid and acetic acid in the total VFA is consistent with the numerical methane reduction ($p = 0.30$) that we observed in this study. According to Russell and Houlihan [39], ionophores act on Gram-positive bacteria, increasing the proportion of Gram-negative bacteria, which are more resistant to these substances, causing an increase in propionic acid and a decrease in methane emissions. Supplementation with 40-AEMO showed a weak tendency on alternate VFA proportion, indicating that AEMO may have a potential methane-mitigating effect. Future studies using a broader range of extract concentrations and direct measurements of methane are warranted to clarify the relationship between rumen fermentation and methane emissions and to better elucidate the effects of AEMO.

Another factor contributing to rumen fermentation alteration theory is the decreased allantoin excretion in the urine. Allantoin is directly linked to microbial protein production, where the greater the microbial synthesis, the greater the allantoin output in the urine [40]. This is evidence that Moringa compounds may impair ruminal fermentation and microbial synthesis. Some studies [41,42] report that certain plant compounds have an antimicrobial effect on the body. Moringa has some of these compounds, such as polyphenols and tannins [43]. The antimicrobial effect of these compounds can lead to rupturing the bacterial cell membrane, disintegrating it, and causing ion leakage [44]. AEMO supplementation

may have influenced ruminal bacteria, as indicated by the numerical reduction in allantoin levels, suggesting a possible antimicrobial effect that could impair ruminal degradation and microbial protein synthesis.

A linear decrease in milk protein yield was observed with AEMO supplementation, suggesting a limitation in amino acid availability for protein synthesis. This restriction in nutrient supply may result from reduced ruminal degradation and a consequent decline in microbial protein synthesis, an important source of amino acids for ruminants and the primary precursors for milk protein synthesis [45]. However, when 40 mL of extract (40-AEMO) was used, milk production was not affected, although it had reduced milk protein. Limitations on protein production may be due to the effect of AEMO on rumen degradation and, consequently, on microbial synthesis, which justifies the lower milk protein production. The amino acid composition of the microbial protein is similar to the amino acid requirement for milk production [46], which indicates that ewes receiving AEMO had a deficiency in amino acid balance. When the animal absorbs a greater quantity of amino acids, it will consequently increase the use of these molecules by splanchnic tissues [47], increasing its catabolism. These events will reduce the efficiency of protein synthesis in milk [48]. Loss can also occur if there is an imbalance in rumen fermentation; this leads to an increase in ammonia, which, when absorbed, is directed to the liver and excreted in the form of urea [49]. The 40-AEMO probably reduced ruminal fermentation and protein efficiency post-absorption since there was a lower milk protein production for treatment 40-AEMO and a weak trend for greater urea urinary excretion. Furthermore, the absence of significant effects may also be attributed to the limited number of experimental units and the inherent variability in animal responses, even though the numerical differences observed were biologically evident.

5. Conclusions

The inclusion of aqueous extract of Moringa oleifera as an additive in lactating ewes does not affect the intake and digestibility of nutrients. Furthermore, it can affect ruminal fermentation and microbial synthesis, with possible alteration of VFA and methane emission and consequently milk protein production. Therefore, we recommend studies with different extract concentrations to investigate possible relationships between rumen fermentation and the synthesis of compounds in milk and use direct methane measurements to test mitigation potential.

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Data Availability Statement: The datasets presented in this article are not readily available because the data are part of an ongoing study. Requests to access the datasets should be directed to the corresponding authors.

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Abbreviations

The following abbreviations are used in this manuscript:

AEMO	Aqueous extract of Moringa oleifera
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic)
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric-reducing antioxidant power
TBARS	Thiobarbituric acid-reactive substances
20-AEMO	20 mL of aqueous extract of Moringa oleifera
40-AEMO	40 mL of aqueous extract of Moringa oleifera
DM	Dry matter
CP	Crude protein
EE	Ether Extract
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
NFC	Non-fiber carbohydrate
iNDF	Indigestible neutral detergent fiber
VFA	Volatile fatty acid

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