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Phytochemical Composition and Effects of Aqueous Extracts from *Moringa oleifera* Leaves on In Vitro Ruminal Fermentation Parameters

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Simple Summary: The bioactive compounds present in natural additives have the potential to modulate the ruminal fermentation process. However, their effects depend on their concentration in these additives. In this study, we analyzed the phytochemical composition, antioxidant activity, and effects on ruminal fermentation of aqueous extracts obtained from *Moringa oleifera* leaves. The extracts were prepared using fresh and dried leaves through three extraction methods: maceration, infusion, and decoction. Extracts from fresh leaves showed a higher concentration of bioactive compounds compared to those obtained from dried leaves. Regarding extraction methods, the maceration and decoction of fresh leaves resulted in lower concentrations of flavonoids and phenolic compounds, respectively. In the context of ruminal fermentation, the use of aqueous extracts from fresh leaves increased the concentration of short-chain fatty acids. Thus, the use of fresh *Moringa oleifera* leaves is recommended for the production of aqueous extracts, due to the higher extraction of bioactive compounds, highlighting their potential as a natural additive in ruminant diets.

Abstract: This study evaluated the phytochemical composition of aqueous extracts of *Moringa oleifera* (MO) obtained by maceration, decoction, and infusion of fresh or dried leaves and their effects on in vitro ruminal fermentation parameters. Phytochemical prospecting analyses were conducted to determine the bioactive compounds in each aqueous extract. Regarding the in vitro ruminal fermentation study, the seven treatments were the following: no addition of extract or control (CON); extract obtained by maceration of fresh leaves (MFL); extract obtained by maceration of dry leaves (MDL); extract obtained by decoction of the fresh leaves (DFL); extract obtained by decoction of dry leaves (DDL); extract obtained by infusion of fresh leaves (IFL) and extract obtained by infusion of dry leaves (IDL). The concentration of all bioactives (saponins, flavonoids, tannins, and alkaloids) quantified was higher when fresh MO leaves were used ($p < 0.001$). DFL and DDL provided less elimination of azino-bis radicals. On the other hand, MFL resulted in a greater elimination of these radicals. Extracts obtained from fresh leaves resulted in a greater total production of short-chain fatty acids, acetate, and butyrate ($p < 0.05$). Compared to the control treatment, the inclusion of extracts obtained from fresh leaves provided a higher concentration of propionate ($p = 0.049$). It is thereby concluded that the use of fresh MO leaves for the production of aqueous extracts is the most recommended, as it results in a higher concentration of bioactive compounds. The use of aqueous extracts of fresh MO leaves increases the total production of fatty acids but does not change their proportion.

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1. Introduction

The use of ruminal modulatory additives in ruminant diets represents the main strategy for manipulating ruminal fermentation and increasing the efficiency of dietary nutrient utilization, as well as reducing energy losses, particularly those related to methane formation and emission. In this context, plant extracts are widely accepted as natural additives capable of manipulating ruminal fermentation, as they contain several bioactive compounds with antimicrobial properties [1].

There is a wide variety of plants with potential use in ruminant nutrition due to the presence of bioactive compounds in their composition that can modulate the fermentation process in the rumen. In this context, *Moringa oleifera* (MO) stands out, a plant which has high biomass production, high nutritional value, the presence of antioxidants, and a high concentration of bioactive compounds [2,3]. Among the bioactive compounds in MO, flavonoids, alkaloids, tannins, and saponins are the main ones [4].

These compounds have already demonstrated antimicrobial activity against Gram-positive bacteria [5,6]. In the rumen, Gram-positive bacteria are mainly responsible for the higher production of acetate and ammonia, which are considered inefficient processes in terms of energy use and nitrogen, respectively [7,8]. Several studies have proven the antioxidant capacity [9] of the aqueous extract of fresh MO leaves and its positive effects on in vitro ruminal fermentation [10,11].

Even with the proven benefits of the use of aqueous extracts from MO, one of the factors that influence the composition of bioactive compounds is the form of extraction, and still, it is not known which extraction form is the most efficient to obtain a higher concentration of bioactive compounds. In this study, we hypothesized that the different extraction methods of dried and fresh MO leaves may influence the phytochemical composition of the aqueous extracts, leading to variations in their antioxidant capacity and their effects on ruminal fermentation parameters.

In this context, the aim was to analyze the phytochemical composition of aqueous extracts obtained by the maceration, decoction, and infusion of fresh or dried MO leaves and the effect of their extracts on the parameters of ruminal fermentation in vitro.

2. Materials and Methods

2.1. Preparation of MO Extracts

The MO leaves were randomly collected from several young and mature trees at the Experimental Farm of the Federal University of Grande Dourados (UFGD), located in the Brazilian state of Mato Grosso do Sul, during spring. The samples were taxonomically identified and deposited in the UFGD Herbarium with no. DDMS8141 (http://ddms.jbrj.gov.br/v2/consulta.php) (accessed on 17 January 2025).

The collected leaves were washed in running water. The excess water was removed, and the leaves were packed in plastic bags duly identified and stored at −20 °C for the subsequent preparation of the extracts. After slowly defrosting in the refrigerator, part of the leaves were dried in a forced air circulation oven at 45 °C for 72 h. Subsequently, the dry leaves were grounded in a Willey knife mill in a 1 mm sieve.

For the preparation of extracts, three extraction methods (infusion, maceration, and decoction) were used from fresh and dried MO leaves, according to Vongsak et al. [12]. In all extraction methods, 76.86 g (corresponding to 20 g of DM) of fresh leaves of MO and 22.49 g (corresponding to 20 g of DM) of dried leaves were weighed, both fractionated

into small pieces. In the infusion process, 200 mL of boiling distilled water was added, which remained for 24 h in the incubator at 30 °C and was then filtered through a Whatman No. 1 filter. For the maceration technique, 200 mL of cold distilled water was added. Subsequently, the material was beaten in a blender for 30 s and filtered in Whatman No. 1 filter. In the decoction method, 200 mL of cold distilled water was added. The material was boiled at 100 °C for 30 min. After this period, the material was removed for cooling for 30 min, being filtered later in Whatman No. 1 filter, and stored in a refrigerator at temperatures between 4 °C and 8 °C. The extracts obtained were submitted to quantitative analyses for the determination of bioactive compounds.

2.2. Phytochemical Composition of the Extracts

The quantification of saponins followed the methodology of Obdoni and Ochuko [13]. For this, 20 mL of aqueous extract was added in a Becker. Then, 100 mL of 20% ethanol was added. The sample was heated in a water bath for 4 h at 55 °C and occasionally stirred. After this, the material was filtered in Whatman No. 42 filter paper. In the residue, 200 mL of 20% ethanol was added. The resulting extract was placed in a water bath at 90 °C. In a separation funnel, 20 mL of diethyl ether was added to the extracts and vigorously stirred. The water layer was stored, and the remaining ether layer was discarded. After this, 60 mL of n-butanol and 20 mL of 5% sodium chloride were added. The remaining solution was heated and dried in a water bath. The dry residue was weighed to determine the saponins in mg/100 mL.

Flavonoids were quantified according to the methodology of Edeoga et al. [14]. In a Becker, 10 g of aqueous extract and 100 mL of 80% aqueous methanol were added. The material was filtered in a Whatman No. 42 (125 mm) paper filter and then placed in a previously weighed crucible and left in a water bath up to 39 °C for 4 h. The dry residue was weighed to obtain the amount of flavonoids in mg/100 mL. Tannin analysis was performed according to Vetter and Barbosa [15]. In a Becker, 100 mL of distilled water and 2 g of extract were added. The solution was kept in a water bath at 90 °C for one hour. Subsequently, the mixture was filtered using Whatman No. 1 paper and the residue was extracted again. A total of 500 mL of distilled water was added to the filtrate. From this solution, 100 mL was transferred to a Becker, and we added 10 mL of 40% formaldehyde and 5 mL of concentrated sulphonic acid. The whole mixture was refluxed for 30 min and allowed to cool. The mixture was filtered, dried, and weighed to determine the total tannin amount in mg/100 mL. The analysis of alkaloids was performed following the methodology described by Harborne [16]. In a Becker, 5 g of aqueous extract and 200 mL of 10% acetic acid in ethanol were added. The sample was covered with plastic insulfilm and remained at rest for 4 h.

After this procedure, it was filtered with Whatman No. 42 filter paper. The extract remained in a water bath until one-quarter of the original volume was left. Subsequently, 15 drops of concentrated ammonium hydroxide were added to the extract. The solution was left to rest for about 4 h. After refiltration in Whatman No. 42 filter paper, the residue was dry and heavy, with the weight representing the amount of alkaloids in mg/100 mL. For the determination of the total phenolic content of the extract, we followed the method of Folin–Ciocalteu [17]. For this, to every 100 mL of methanol extract (1 g/L) were added 1 mL of distilled water and 0.5 mL of Folin–Ciocalteu reagent (v/v 1:10). After 3 min, 1.5 mL of a saturated sodium carbonate solution (2%) was added. The mixture was left to stand for 30 min, and then the optical density was measured by absorbance at 765 nm, using a spectrophotometer. The quantification was performed based on a standard curve of gallic acid prepared in 80% methanol, and the result was expressed in mg TAE/100 g.

2.3. Antioxidant Activity

As for the antioxidant activity, extracts were analyzed using quercetin as a positive comparison. The plates were diluted in 10% chloroform/methanol and, after drying, they were nebulized with a 0.4 mmol/L solution of 1,1-diphenyl-1-picril-hydrazil (DPPH) in methanol. The cells were observed until the appearance of yellow spots on a purple background, indicating possible antioxidant activity [18]. The activity of sequestering free radicals from the MO aqueous extracts was determined by the DPPH free radical method [19]. Several concentrations of the samples were added to 2 mL of DPPH (0.1 mM) methanol solutions prepared daily. The mixture was stirred and left to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm. Butyl hydroxytoluene (BHT) was used as a positive control. Sample concentrations for 50% DPPH inhibition (IC50) were calculated based on the graph of I% (percentage of inhibition) versus a concentration of the sample in micrograms per milliliter (μg/mL). The antioxidant activity was calculated using the azino-bis (ethyl-benzothiazoline-6-sulfone acid; ABTS) radical elimination method. The extracts (1.0 mg/mL each) were diluted to reach final concentrations of 250, 125, 50, 25, 10, and 5 μg/mL in methanol. To form the cation radical (ABTS), 7.0 mM of ABTS and 140 mM of potassium persulphate were mixed and kept in the dark for 16 h at room temperature.

Before use, this solution was diluted to achieve an absorbance of 0.700 ± 0.05 at 734 nm using ethanol (P.A.). For sample analysis, 3 mL of this solution was added to 30 μL of different concentrations of the methanol sample (5–250 μg/mL). After 30 min, the absorbance was obtained at 734 nm using a spectrophotometer. The IC50 value was calculated as the sample concentration needed to inhibit 50% of free radicals, graphically representing the I% versus the concentration of the extract.

2.4. In Vitro Experiment

Incubation

The in vitro ruminal incubation methodology adapted from Goering and Van Soest [20] was used. Before starting the incubation, ruminal fluid was collected from a fistulated adult cattle, with an approximate average weight of 450 kg, fed exclusively on pasture (*Urochloa decumbens*). For incubation, 96 Erlenmeyers were used, each with a capacity of 125 mL. The seven treatments, with three replications (incubation rounds) each, were as follows: no addition of extract or control (CON); extract obtained by maceration of fresh leaves (MFL); extract obtained by maceration of dry leaves (MDL); extract obtained by decoction of fresh leaves (DFL); extract obtained by decoction of dried leaves (DDL); extract obtained by infusion of fresh leaves (IFL); and extract obtained by infusion of dry leaves (IDL).

In the Erlenmeyers, 0.5 g of substrate (alfalfa hay; Table 1) was added into a non-woven tissue bag (TNT; 5 × 5 cm), alongside 10 mL of ruminal fluid, 40 mL of mineral buffer solution, and 0.4 mL of aqueous extract. For the control treatment, the same volume of distilled water was added. The buffer solution used in this research consisted of solutions A and B, which were mixed at a 1:5 ratio to reach a pH of 6.8. The following reagents were used for the preparation of these solutions:

Solution A: potassium phosphate monobasic (KH_2PO_4), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), sodium chloride (NaCl), calcium chloride ($CaCl_2 \cdot 2H_2O$), and urea.

Solution B: sodium carbonate (Na_2CO_3) and sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$).

This procedure was performed under constant spraying of CO_2, and, immediately after inoculation, the bottles were sealed with rubber stoppers.

The Erlenmeyers were incubated in a BOD-type oven at 39 °C for a period of 0, 3, 6, 12, 24, and 48 h. After each incubation time, the Erlenmeyers were placed on ice to stop the ruminal fermentation process. From the liquid fraction contained in each Erlenmeyer, the

pH was measured. Subsequently, 1 mL of the post-incubation ruminal fluid was transferred to an Eppendorf containing 1 mL of formaldehyde (37%) and then stored in a refrigerator for subsequent protozoal counting. For the analysis of short-chain fatty acids (SCFAs), 2 mL of ruminal fluid was stored in an Eppendorf containing 20 µL of sulfuric acid (50%) and then frozen. The remaining liquid was frozen for further evaluation of ammoniacal nitrogen (N-NH_3).

Table 1. Chemical composition of alfalfa hay.

Nutrient	Quantity (%)
Dry Matter	89.81
Mineral Matter	9.10
Crude Protein	17.43
Ether Extract	4.06
Neutral Detergent Fiber	55.20
Acid Detergent Fiber	34.24

2.5. Chemical Analysis

The bags taken from the Erlenmeyers were washed with distilled water until the water was transparent. Immediately after, the bags were pre-dried in a forced air circulation oven at 65 °C for 24 h and then dried in a final drying oven at 105 °C for 2 h. After this, the bags were placed in a dryer for 30 min and then weighed. The dry matter degradability (DMD_{eg}) was estimated by the difference in weight of the bags before and after incubation. For the determination of the degradability of the neutral detergent fiber ($NDFD_{eg}$), NDF analysis was performed according to the methodology proposed by Van Soest et al. [21] from the bags obtained from in vitro fermentation.

The analysis of N-NH_3 was performed according to the technique described by Fenner [22] and adapted by Vieira [23]. For protozoan counting, 2 mL of rumen fluid was preserved in 2 mL of formalin (18.5%). These samples were stored at 2 °C until protozoan counting was performed using a microscope [24]. For the determination of SCFA, 1.6 mL ruminal fluid was centrifuged at 15,000× *g* for 15 min at 4 °C with 0.4 mL of a 3:1 solution of 25% metaphosphoric acid and 98–100% formic acid and 0.2 mL of 100 mM (internal standard) 2-methyl-butyric acid solution. After centrifugation, approximately 1.2 mL was transferred to a chromatographic flask. From this volume, 1 µL was automatically injected by the gas chromatograph injector system (CG HP 7890A; Injector HP 7683B, Agilent Technologies, Centerville Road, Wilmington, DE, USA), equipped with capillary column HP-FFAP (1909F-112; 25 m; 0.32 mm; 0.5 µm; JeW Agilent Technologies, Centerville Road, Wilmington, DE, USA). The drag gas used was He, maintained at a linear speed of 42 cm/s. The temperatures of the injector and detector were, respectively, 250 °C and 300 °C, and the initial temperature of the capillary column was 40 °C. The concentration of SCFAs (mM) was determined based on an external calibration curve.

2.6. Experimental Design and Statistical Analysis

The evaluation of the phytochemical composition of the extracts was conducted in a randomized block design, where each repetition of the extraction process consisted of one block, in factorial scheme of 2 × 3, with two ways of using the leaves (dry and fresh) and three extraction methods (infusion, decoction, and maceration). The statistical analyses were performed with the SAS statistical program (SAS Institute Inc., Cary, NC, USA), considering the effect of leaves (dry and fresh), extraction methods (infusion, decoction, and maceration), and the interaction between the methods and the leaves. The averages were compared by Tukey's test at 5% probability.

The in vitro ruminal fermentation assay was conducted in a randomized block design, considering each incubation round as a block, in a factorial scheme with an additional treatment (2 × 3 + 1), with two ways of using the leaves (dry and fresh), three extraction methods (infusion, decoction, and maceration), and a control treatment. The statistical analyses were carried out with the help of the SAS statistical program (SAS Institute Inc., Cary, NC, USA), considering the effect of leaves (dry and fresh), extraction methods (infusion, decoction, and maceration), and the interaction between the methods and the leaves. The averages were compared with Tukey's test at 5% probability. The treatment that generated better results was compared to the control treatment.

3. Results

3.1. Bioactive Compounds and Antioxidant Activity of Extracts

According to the analyses related to the phytochemical composition, all aqueous extracts of MO leaves presented the following bioactive compounds: saponins, flavonoids, tannins, alkaloids, and total phenols. In addition, all the extracts showed antioxidant activities against DPPH and azino-bis radicals (ABTS; Table 2).

Table 2. Bioactive compounds and antioxidant activity of aqueous extracts of *Moringa oleifera* as a function of leaf condition and extraction methods.

Item	Dry Leaves			Fresh Leaves			SEM	*p* Value		
	Maceration	Infusion	Decoction	Maceration	Infusion	Decoction		Leaves	Method	L*M
Saponins (mg/100 mL)	1.73	1.74	1.76	5.11	5.16	5.14	0.066	<0.001	0.698	0.860
Flavonoids (mg/100 mL)	4.09	4.27	4.18	12.14	12.56	12.41	0.079	<0.001	<0.001	0.066
Tannins (mg/100 mL)	8.29	8.55	7.99	22.41	23.05	21.46	0.741	<0.001	0.078	0.500
Alkaloids (mg/100 mL)	3.57	3.53	3.54	10.42	10.25	10.41	0.075	<0.001	0.064	0.204
Total Phenols (mg TAE/100 g)	100.10	102.00	98.00	105.00	108.95	104.00	0.069	<0.001	0.008	0.698
DPPH (IC_{50} µg/mL) [1]	51.00	50.58	48.00	57.00	57.13	60.12	0.087	<0.001	0.979	0.054
ABTS (IC_{50} µg/mL) [2]	68.00 b	55.25 c	53.00 d	79.00 a	60.12 cd	50.20 d	0.097	<0.001	<0.001	<0.001

SEM: standard error of the mean; and L*M: interaction between leaf condition and extraction method. Different letters on the same line demonstrate differences between treatments due to the interaction between the leaf condition and the method. [1] Oxidation ability to consume the radical DPPH; and [2] oxidant activity comprising the elimination of azino-bis radicals (ABTS).

It was observed that the content of bioactive compounds was higher ($p < 0.001$) in fresh leaves. As for the extraction method, it was found that only flavonoids, total phenols, and ABTS were influenced by this factor ($p < 0.05$). Regardless of the leaves' condition, maceration resulted in lower values of flavonoids, while decoction generated lower values of total phenols. There was an effect of the interaction between the condition of the leaves and the extraction method on the antioxidant activity (ABTS) ($p < 0.001$). The decoction of fresh and dry leaves provided lower values of ABTS, while ABTS was higher after the maceration of fresh leaves.

3.2. In Vitro Incubation

There was no interaction between treatments and incubation time affecting the in vitro ruminal fermentation parameters ($p > 0.05$). Aqueous MO extracts did not influence DMD_{eg} ($p > 0.05$). It was observed that, in all treatments, there was an increase in DMD_{eg} as a function of incubation times (Figure 1).

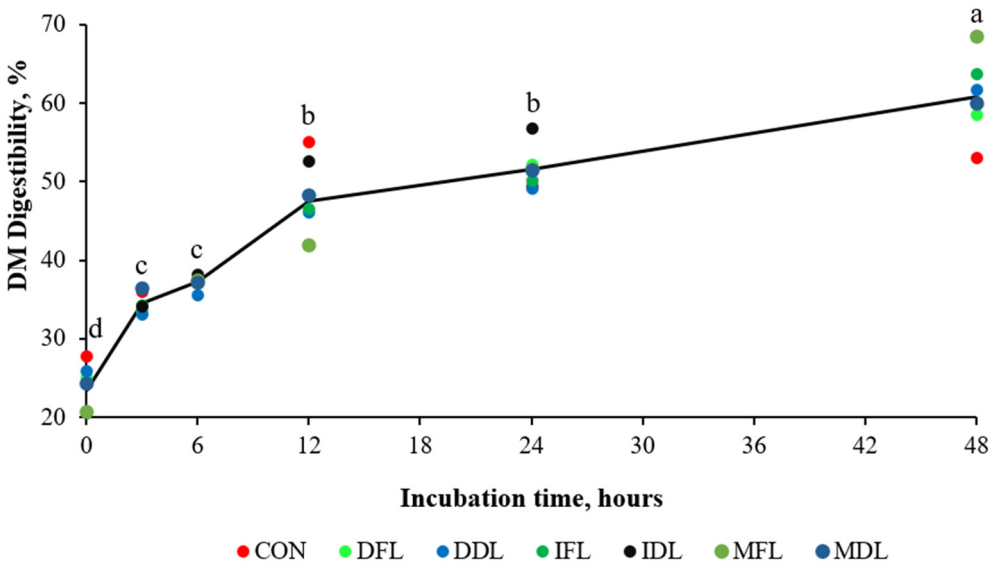

Figure 1. In vitro degradability of the substrate DM as a function of incubation time. CON = control treatment; DFL = decoction of fresh leaves; DDL = decoction of dry leaves; IFL = infusion of fresh leaves; IDL = infusion of dry leaves; MFL = maceration of fresh leaves; and MDL = maceration of dry leaves. Different letters represent significant differences in time ($p < 0.05$).

$NDFD_{eg}$ was not affected by the treatments evaluated ($p > 0.05$). However, all treatments were influenced by the incubation time. The lowest values for $NDFD_{eg}$ were observed at 0 h (16.53%), while the highest values were recorded after 48 h (46.19%; Figure 2). The pH was not influenced by the different aqueous extracts of MO ($p > 0.05$). However, the incubation times affected the mean pH values (Figure 3).

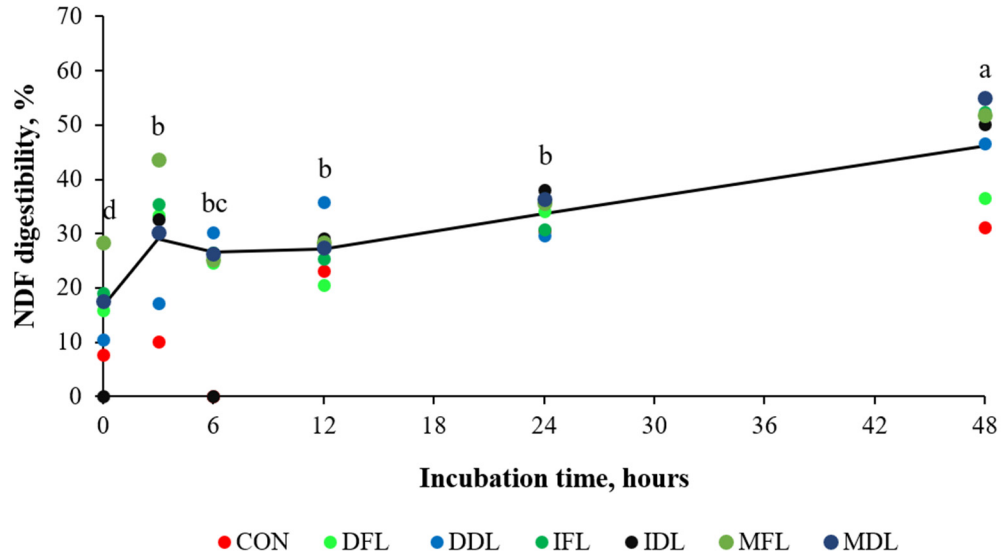

Figure 2. In vitro degradability of the substrate NDF as a function of the incubation time. CON = control treatment; DFL = decoction of fresh leaves; DDL = decoction of dry leaves; IFL = infusion of fresh leaves; IDL = infusion of dry leaves; MFL = maceration of fresh leaves; and MDL = maceration of dry leaves. Different letters represent significant differences in time ($p < 0.05$).

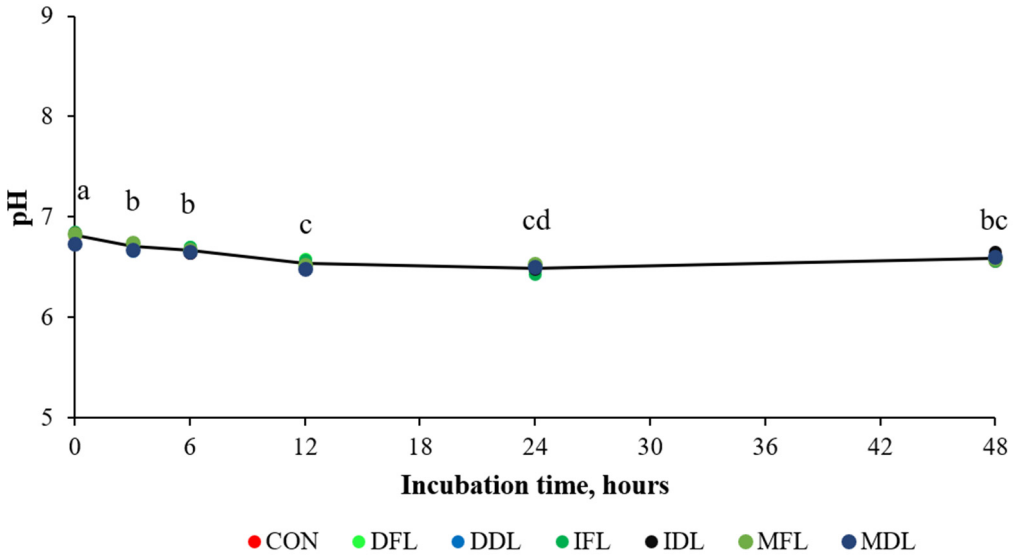

Figure 3. Ruminal pH values as a function of incubation time. CON = control treatment; DFL = decoction of fresh leaves; DDL = decoction of dry leaves; IFL = infusion of fresh leaves; IDL = infusion of dry leaves; MFL = maceration of fresh leaves; and MDL = maceration of dry leaves. Different letters represent significant differences in time ($p < 0.05$).

There was no effect of the treatments on the concentration of N-NH_3 ($p > 0.05$). However, the incubation time resulted in changes in N-NH_3 concentration. The mean values increased from 0.72% at 0 h to 0.98% at 3:00 and 1.00% at 6:00. At 48:00, the concentration of N-NH_3 was observed to be 0.95% (Figure 4). The protozoan population was not affected by the different extracts ($p > 0.05$). However, there was an alteration in the protozoa population over the incubation time ($p < 0.05$). The initial count (0 h) was 3.71×10^4, and the final count (48 h) was 4.02×10^4 (Figure 5).

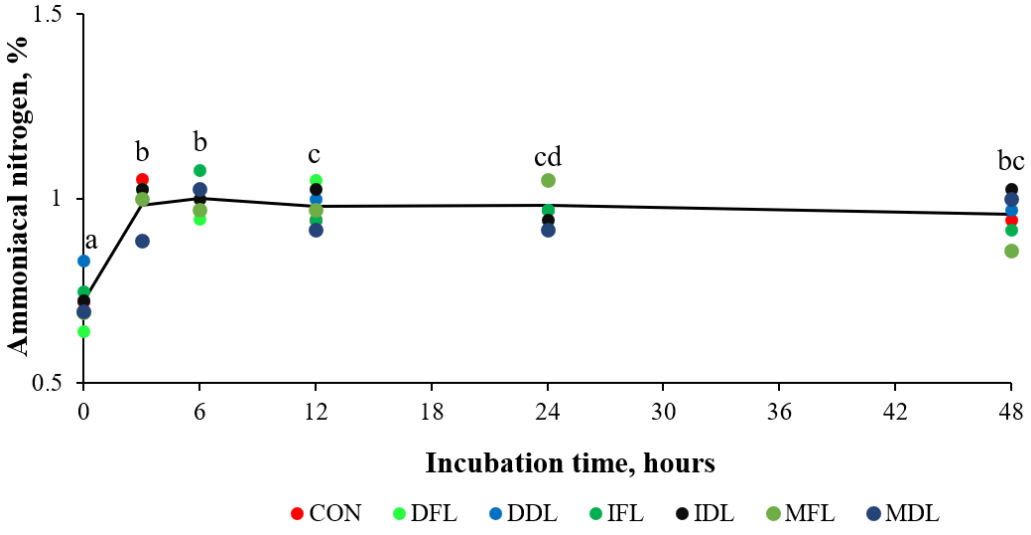

Figure 4. Ammoniacal nitrogen values as a function of incubation time. CON = control treatment; DFL = decoction of fresh leaves; DDL = decoction of dry leaves; IFL = infusion of fresh leaves; IDL = infusion of dry leaves; MFL = maceration of fresh leaves; and MDL = maceration of dry leaves. Different letters represent significant differences in time ($p < 0.05$).

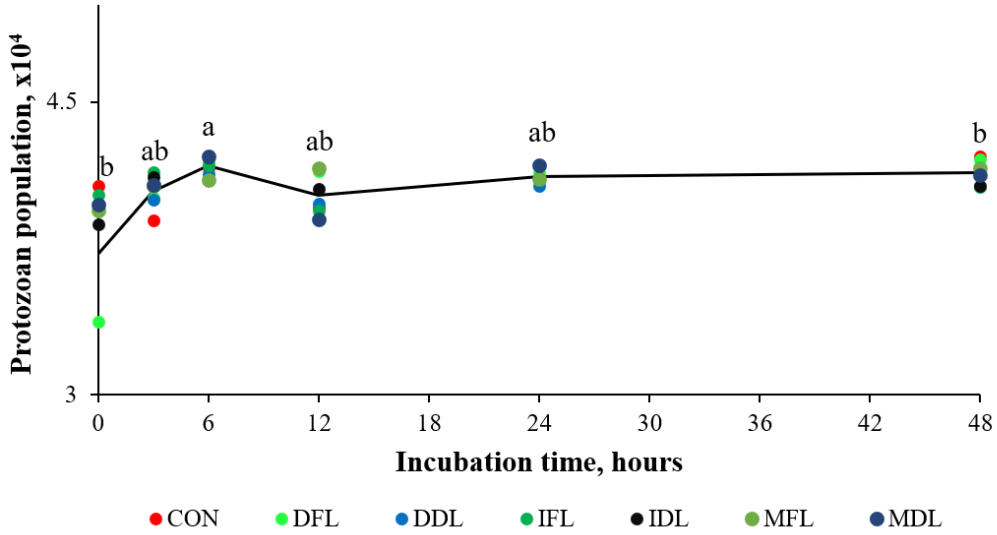

Figure 5. Protozoan population as a function of incubation time. CON = control treatment; DFL = decoction of fresh leaves; DDL = decoction of dry leaves; IFL = infusion of fresh leaves; IDL = infusion of dry leaves; MFL = maceration of fresh leaves; and MDL = maceration of dry leaves. Different letters represent significant differences in time ($p < 0.05$).

The extracts obtained from MO leaves resulted in a higher total production of SCFA, acetate, and butyrate ($p < 0.050$; Table 3). Compared to the control treatment, the inclusion of extracts obtained from fresh leaves provided a higher concentration of propionate ($p = 0.049$). In addition, the total concentration of SCFA, acetate, and butyrate also showed higher values with fresh leaf extracts compared to the control treatment ($p < 0.05$).

Table 3. Profile of short-chain fatty acids after 24 h in vitro incubation depending on the different aqueous extracts of *Moringa oleifera* Lam.

Item	Control	Dry Leaves			Fresh Leaves			SEM	*p* Value			
		Maceration	Infusion	Decoction	Maceration	Infusion	Decoction		Leaves	Method	L*M	FL*C
Acetate, mM	9.49	10.21	9.03	10.65	17.01	14.95	13.36	3.120	0.029	0.714	0.644	0.030
Propionate, mM	3.37	4.69	3.83	4.13	7.26	5.45	5.41	1.437	0.070	0.409	0.812	0.049
Butyrate. mM	1.10	1.19	0.86	1.10	1.78	1.49	1.54	0.389	0.049	0.553	0.937	0.060
Isovaleric, mM	0.22	0.24	ND	0.24	0.20	0.24	0.23	0.030	0.576	0.737	0.693	0.843
Valeric, mM	ND	0.27	ND	ND	0.21	0.27	0.20	0.003	0.058	0.072	ND	ND
Total, mM	14.19	16.47	13.71	16.00	26.35	22.15	20.53	4.871	0.035	0.568	0.736	0.036
Acetate, %	66.88	62.16	65.00	66.47	64.57	67.58	65.50	2.908	0.455	0.358	0.642	0.590
Propionate, %	23.77	28.45	28.87	26.03	27.50	24.70	25.90	3.595	0.431	0.740	0.717	0.267
Butyrate, %	7.78	7.19	6.14	6.78	6.75	6.72	7.49	0.656	0.482	0.346	0.448	0.044
Isovaleric, %	1.56	1.59	ND	1.42	0.76	0.95	1.41	0.567	0.478	0.929	0.576	0.129
Valeric, %	ND	1.22	ND	ND	0.81	1.05	0.81	0.032	0.066	0.156	ND	ND
Acetate: Propionate	2.82	2.18	2.33	2.59	2.37	2.74	2.55	0.417	0.469	0.587	0.756	0.305

SEM: standard error mean; ND: not determined; L*M: interaction between leaf condition and extraction method; and FL*C: comparison between the mean values of fresh leaf (FL) condition and control (C) treatment.

4. Discussion

4.1. Phytochemical Composition and Antioxidant Activity

According to the present study, MO is an interesting source of saponins, flavonoids, tannins, and alkaloids, and the concentration of these bioactive compounds is affected by the condition of the leaves (dry and fresh). The lower content of bioactive compounds present in extracts produced from dried leaves indicates that drying reduces the quality of the extracts. Several factors can influence the concentration of these compounds, among which are environmental conditions, harvest time [25], plant genetics [26,27], drying method [28,29], leaf maturation stage [30], plant parts [31], leaves [32], and the extraction method used [33,34]. Nobósse et al. [35], evaluating the phytochemical content of aqueous extract from fresh MO leaves, observed that the total phenolic content varied between 2.1 and 3.6 g GAE/100 g DM, and the flavonoid content ranged between 0.9 and 1.0 g

CE/100 g DM, depending on the age of the leaves. The extraction method affected the concentration of total phenols, whereby decoction resulted in lower values of these bioactive compounds. Decoction extraction involves the use of high temperatures, which decreases the concentration of phenolic compounds, which are heat-sensitive [36].

In this study, the extracts produced from fresh leaves showed greater ABTS radical elimination. This result is due to the higher concentration of flavonoids in these extracts. According to Nobósse et al. [35], the elimination of DPPH is negatively affected by the presence of flavonoids, which is due to the transfer of hydrogens from these bioactive compounds to free radicals. With the decoction of fresh and dry leaves, the elimination capacity of ABTS was reduced. This was probably due to the lower concentration of phenolic compounds in this extraction method. Phenolic compounds are the main ones responsible for the elimination of ABTS [35]. The high levels of antioxidant activity attributed to phenolic compounds suggest that they can be used as supplements to prevent oxidative stress-related problems.

Cohen-Zinder et al. [37] found that milk from cows fed with MO had higher antioxidant activity compared to the control treatment without the use of MO. This demonstrates that the use of MO in ruminant diets may improve the oxidative status and organoleptic characteristics of the products derived from these animals.

4.2. Parameters of Ruminal Fermentation in Vitro

In the present study, the inclusion of different aqueous extracts of MO did not affect DMD_{eg} and $NDFD_{eg}$. Leitanthem et al. [38] observed an increase in dry matter degradability when they included increasing levels of MO extract in an in vitro ruminal fermentation assay. Kholif et al. [39], when evaluating increasing levels (0, 10, 15, and 20%) of MO leaf powder (LPMO) in the diet of goats, found that the inclusion of 15% LPMO resulted in higher DMD_{eg} and $NDFD_{eg}$ values. Similarly, Kholif et al. [40] observed that DMD_{eg} increased as a function of increasing levels (10, 20, and 40 mL) of aqueous MO leaf extract in the diet of goats. Singh et al. [41] explains that the influence of plant bioactive compounds such as tannins and saponins on these parameters depends on the dose of extract used and the chemical structure of the bioactive compounds. In this sense, likely, the concentrations of bioactive compounds in the doses of the extracts used in this study may not have been sufficient for the appearance of effects on degradability.

However, the values for DMD_{eg} and $NDFD_{eg}$ were increased with a longer incubation time. This is justified by the fact that longer incubation times allow for a greater activity of the microorganisms involved in the ruminal fermentation process, reflected in a greater degradation of substrates.

Due to the antimicrobial properties of the bioactive compounds present in MO, especially in relation to Gram-positive bacteria, it was expected that the inclusion of extracts from fresh leaves would result in a lower acetate production and a higher propionate concentration. However, in this work, we observed increased acetate production, while the propionate concentration was not changed. When evaluating the use of moringa root bark as a replacement for sodium monensin in lamb diets, Soltan et al. [42] observed that moringa root bark led to increased acetate production in the rumen. Kholif et al. [39] found that the inclusion of 15% LPMO in the diet of goats resulted in higher propionate concentration, without changing the concentration of acetate and butyrate. In another study, the concentration of acetate and propionate increased linearly as a function of increasing levels of aqueous MO extract in the diet of dairy goats [40]. In buffaloes, Abdel-Raheem and Hassan [43] observed higher concentrations of acetate and propionate with 15% LPMO in the diet.

The total production of fatty acids was also increased after the inclusion of extracts from fresh leaves. This was mainly due to the higher acetate production, suggesting that the bacteria producing acetate were benefited by the inclusion of these extracts. This behavior reinforces the theory that the doses used here were low and that the bioactive compounds present in MO may have been partially degraded by the ruminal microorganisms, resulting in an increased production of SCFA. The aqueous extracts of fresh MO leaves increased the propionate concentration compared to the control treatment. This result has great nutritional relevance, since propionate is the main glucose precursor for ruminants [44].

5. Conclusions

The use of fresh leaves of *Moringa oleifera* Lam. for the production of aqueous extracts is the most recommended, as it results in a higher concentration of active bioactive compounds. Among the extraction methods, decoction is less indicated due to its negative effects on the concentration of phenolic compounds and antioxidant activity. The maceration extraction method preserves the concentration of bioactive compounds and antioxidant activity of fresh leaves of *Moringa oleifera* Lam. As for the in vitro ruminal parameters, the use of aqueous extracts of fresh leaves of *Moringa oleifera* Lam. increases the total production of fatty acids but does not change the proportion of fatty acids.

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