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Article

Effect of *Moringa oleifera* in *in vitro* rumen fermentation tests and its impact on greenhouse gases

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Abstract:

The rumen environment has been biotechnologically manipulated to improve animal productivity and natural, healthy, and ecological alternatives have recently been sought for this purpose. *Moringa oleifera* has been tested as livestock feed because its leaves are rich in minerals, protein, and secondary metabolites. The objective was to evaluate the changes in rumen activity due to the presence of *M. oleifera*. Three treatments were tested: CA (control 100 % alfalfa), LM (15 % Moringa-85 % alfalfa), and HM (30 % Moringa-70 % alfalfa). The experiment was performed *in vitro* with sheep rumen fluid. No differences were observed ($P>0.05$) for pH and ammoniacal nitrogen ($N\text{-}NH_3$). Dry matter digestibility differed ($P<0.05$) between treatments. Gas production showed differences ($P<0.05$) among treatments. There were differences ($P<0.05$) for the concentration of volatile fatty acids (VFAs). CO_2 and CH_4 were different between treatments ($P<0.05$), with LM being the lowest for both variables. It is concluded that adding moringa to a ration of alfalfa has no effect on pH and $N\text{-}NH_3$; nevertheless, it increases the digestibility of the dry matter and decreases the concentration of VFAs and the digestibility of fibers. In addition, including 15 % of moringa

in a ration of alfalfa can reduce the production of greenhouse gases. It is recommended to continue evaluating this alternative for animal nutrition.

Keywords: *Moringa oleifera*, Rumen fermentation, *Ovis aries*, Greenhouse gases.

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Introduction

Global demand for animal products has increased in recent years[1]. Due to the constant increase in population, there has been a search for alternatives that produce a greater quantity of food of better protein quality and with greater efficiency. In extensive livestock farming, native grasses are used as the main source of food and during the dry period, it is necessary to use feed supplements because the quality of these grasses decreases, which increases feed costs. Therefore, farmers are looking for food sources that are accessible both in terms of their availability and low cost and that do not imply competition with humans[2].

On the other hand, ruminants produce greenhouse gases, such as methane (CH_4), which is emitted into the atmosphere[1,3]. The use of shrubs and trees in animal nutrition can be an alternative to increase digestibility, improve the nutritional value of the diet, and reduce methane production[4]. There is research focused on proving that the use of leaves from various plants, as well as the foliage of some trees, can serve as food, with adequate nutrients to achieve an economically and ecologically viable production that reduces the production of greenhouse gases[1]. Moringa (*Moringa oleifera*) forage is one of these plants[2], as it is characterized by its ease of propagation and low demand for nutrients and water in its growth[5]. It has been shown that it can be a good source of protein, which may represent an alternative to replace conventional protein inputs in ruminants[6].

Moringa oleifera is a thin, evergreen, deciduous tree native to India that has spread to other parts of the world. Worldwide, it is one of the fastest growing trees that has a high biomass yield, high crude protein content (>25 %), and a balance of other nutrients in its leaves[7]. It is also considered one of the most useful trees in the world as all its parts can be used as food, as medicine, or for industrial purposes[8,9,10]. The different parts of *M. oleifera* contain important minerals and are a good source of protein, vitamins, and amino acids. It has also been reported that it has beneficial agronomic properties, such as drought tolerance, high

biomass production, and high crude protein content, which makes it competitive with high-quality forages[8,11], in addition to the fact that its leaves contain many active compounds, such as flavonoids, saponins, tannins, and alkaloids[8]. *M. oleifera* has been used to feed cattle as the leaves are rich in minerals essential for weight gain and milk production, and because it is an excellent source of protein that can improve microbial protein synthesis in the rumen[12]. Its nutritional effect was also analyzed in sheep, where they had an improvement in milk production and quality[12]. However, no studies have been reported on the use of moringa grown in the state of Chihuahua and the impact of its use as feed on animal production. Therefore, this study aimed to evaluate the effect of *Moringa oleifera* leaves grown in the central region of the state of Chihuahua on different parameters of *in vitro* rumen fermentation in order to have a first approach to the viability of using this shrub as feed for cattle in this region.

Material and methods

This study was carried out at the Animal Nutrition Laboratory of the Faculty of Zootechnics and Ecology of the Autonomous University of Chihuahua (UACh, for its acronym in Spanish), located at Km 1 of the Periférico Francisco R. Almada in the city of Chihuahua, Chihuahua, Mexico (28°35′10.9″ N; 106°6′26.6″ W; altitude 1,440 m asl).

Moringa supplement

It was obtained from a plantation of *M. oleifera* trees located in the central region of the state of Chihuahua, Mexico. The leaves of the plant were collected, dried in the shade, and then macerated to obtain powdered feed[13].

Characterization of the bromatological profile

The bromatological characterization of *M. oleifera* leaves and alfalfa (control treatment) was carried out by means of a proximate analysis. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured using the method described by Van Soest *et al*[14] adapted for the Ankom 200 Fiber Analyzer equipment (Ankom Technology, Fairport NY). Crude protein (CP), dry matter (DM), ash, and EE were determined according to the standard

methods of the Association of Official Analytical Chemistry[15]. Minerals were analyzed by atomic absorption and the amino acid profile by chromatography.

Animals and diet

Rumen fluid was obtained from three rumen-fistulated sheep of the Pelibuey breed (45 ± 3 kg). Prior to the fistulation process, the animals were vaccinated (Lapibac; Lapisa®) and dewormed (Levax ADE; BioZoo®). They were then adapted to the diet (alfalfa forage) for 15 d. Feed was offered twice a day (0800 and 1700 h). Once the adaptation period was over, the animals were kept fasting for 24 h and then rumen fluid samples were taken from the three animals directly from the cannula to perform *in vitro* fermentation tests.

Treatments and experimental design

Once the bromatological profile of *M. oleifera* was characterized (Table 1), three treatments were established, looking for isoprotein values; CA (Control: 100 % alfalfa), LM (Low Moringa: 15 % moringa and 85 % alfalfa) and HM (High Moringa: 30 % moringa and 70 % alfalfa). A completely randomized design with repeated measurements over time was used in the *in vitro* fermentation test. There were three replications for each fermentation time (6, 12, 24, and 48 h). The gas production analysis was performed according to Theodorou's[16] technique; to this end, 2 g of the substrate was weighed directly in 100 mL glass tubes with butyl stopper and agraffe. To analyze the rest of the variables studied, 0.5 g of the substrate was weighed in FN57 (Ankom™) bags with a pore size of 25 μm and then each bag was sealed and placed in 250 mL bottles. The rumen microbial inoculum was prepared using two parts of a buffer solution[17] and one part of rumen fluid. Rumen fluid was collected from the three previously fistulated Pelibuey sheep, directly from the cannula before feeding. The inoculum was filtered with muslin and dispensed under CO_2 anaerobiosis conditions (15 mL for gas production and 40 ml for the rest of the parameters); it was immediately sealed and placed in an incubator with stirring at 120 rpm and controlled temperature at 39 °C. Three substrate-free replications were prepared as a control for gas production.

Total gas production was measured with a FESTO® pressure transducer (SIEMENS). The pH was measured with an electronic potentiometer immediately after sampling. The digestibility of NDF and ADF was evaluated using the IVTD - Daisy method (*in vitro* True Digestibility Method)[18]. NDF and ADF were determined at the end of each incubation time; processing was performed in the Ankom® 2000 fiber analyzer according to the methods

described by Van Soest *et al*[14]. N-NH_3 was calculated by spectrophotometry[19]. Volatile fatty acids (VFAs) were determined by gas chromatography with flame ionization detection. A Claurus 400® gas chromatograph (Perkin Elmer) adapted with a Varian CP-wax58 (FFAP) CB capillary column (15 m × 0.53 mm, 0.5 μm) was used[20]. The determination of CH_4 and CO_2 was calculated from the concentrations of VFAs using the equation method proposed by Wolin[21].

Statistical analysis

The data were analyzed with a completely randomized design using the GLM procedure (SAS ver. 9.4); repeated measurements over time were considered for the pH and GP variables and the analysis was performed using the Proc MIXED of SAS version 9.4[22].

Results and discussion

Bromatological analysis of Moringa supplement

The results of the bromatological analysis performed on the moringa supplement are described in Table 1.

Table 1: Bromatological analysis of *Moringa oleifera* supplement

Variable	%
Dry matter	93.87
Ash	15.68
Ethereal extract	5.99
ADF	14.85
NDF	26.42
Crude protein	16.97
Ca	3.23
P	0.27
Mg	0.8
K	1.35
Mn, ppm	120.4
Cu, ppm	18.64
Zn, ppm	20.63

The values obtained differ from what has been reported since the CP was lower and the EE content was higher (Table 1) than reported in other studies[23-27]. The CP content had an acceptable value (16.97 %) for inclusion in the diet of ruminants at different feeding stages (NRC), but it is lower compared to alfalfa[28], which is one of the main hayed forages used in dairy cattle diets. Nonetheless, in northern Mexico, it is common to use oat hay in beef cattle feed, suggesting that moringa may represent an alternative to the use of oat hay, which has a lower CP value[28]. The ash content was higher (15.68 %) than that reported in other studies[8,11,23]. This suggests that dried moringa leaves contain a considerable amount of them. Calcium, magnesium, copper, and potassium had higher values compared to those reported in the literature[25,27,29]. The value of calcium was even higher than that reported for oat and alfalfa hay[30]; this gives an important value to *M. oleifera* since this mineral is of great importance for the regulation of different processes[31], productive aspects, and for the maintenance of bone and dental structure[29].

The amino acid profile found in the moringa supplement is presented below (Table 2).

Table 2: Amino acid content profile of *Moringa oleifera* supplement

Amino acid	**g/100 g**
Alanine	0.87
Glycine	0.63
Valine	0.53
Leucine	0.56
Isoleucine	0.52
Threonine	0.54
Serine	0.76
Proline	0.69
Aspartate	1.93
Methionine	0.11
Glutamate	6.05
Phenylalanine	0.75
Lysine	0.43
Histidine	0.53
Tryptophan	0.27
Cysteine	0.04

Glutamate, aspartate, alanine, leucine, and serine had a higher concentration than reported[32]. On the other hand, the alanine content was similar and leucine content was lower than that found in the literature[32]. However, the data reported by other studies[1,5,8,25] are inconsistent. All amino acid values are below those reported by these authors, except for glutamate and aspartate; this could be due to the fact that the moringa they analyzed was grown in conditions

of humidity, altitude, and temperature very different from those found in the growing area of *M. oleifera* used in this study; therefore, the biochemical behavior of the plant could have generated different concentrations of these metabolites. Although these amino acids, with the exception of leucine, are non-essential, they do represent a good source of nitrogen for microbial metabolism and, therefore, for microbial protein synthesis[33].

Nutritional composition of treatments

The nutritional composition of the three treatments used in *in vitro* fermentation is shown in Table 3.

Table 3: Composition of diets used in *in vitro* fermentation tests

Variable (%)	CA	LM	HM
Dry matter	89.4	90.1	90.7
Ash	11	11.7	12.4
Ethereal extract	2.08	2.7	3.3
ADF	29	26.9	24.8
NDF	36.1	34.6	33.2
Crude protein	21.2	20.6	19.9
Ca	1.4	1.7	1.9
P	0.26	0.3	0.3
Mg	0.32	0.4	0.5
K	3.03	2.8	2.5

CA= control treatment (100 % alfalfa); LM= low moringa treatment (15 % moringa 85 % alfalfa); HM= high moringa treatment (30 % moringa 70 % alfalfa).

In vitro rumen fermentation

It was performed with the three treatments described above. The results of the variables evaluated from *in vitro* rumen fermentation are shown in Table 4.

Table 4: Variables evaluated in the *in vitro* fermentation tests

Variable	CA	LM	HM	*P*-Value
pH	6.74 [a]	6.86 [a]	6.76 [a]	≥ 0.05
DM digestibility, %	63.88 [a]	66.65 [ab]	67.87 [b]	<0.05
NDF, %	24.64 [a]	30.96 [ab]	32.09 [b]	<0.01
ADF, %	17.80 [a]	22.53 [ab]	23.54 [b]	<0.01
GP	49.06 [a]	51.18 [ab]	55.93 [b]	<0.05
Acetic acid, mmol/L	63.9 [a]	42.3 [b]	31.2 [c]	<0.05
Propionic acid, mmol/L	30.6 [a]	21.0 [b]	11.1 [c]	<0.05
Butyric acid, mmol/L	9.3 [a]	4.6 [b]	4.2 [b]	<0.05
TVFAs, mmol/L	103.9 [a]	67.9 [b]	46.5 [c]	<0.05
CO_2 production, % molar	51.7 [a]	48.5 [b]	51.9 [a]	<0.05
CH_4 production, % molar	28.6 [a]	26.0 [b]	32.3 [c]	<0.05
NH_3, mmol/ml	14.6 [a]	14.9 [a]	15.1[a]	>0.05

CA= control treatment (100 % alfalfa); LM= low moringa treatment (15 % moringa 85 % alfalfa); HM= high moringa treatment (30 % moringa 70 % alfalfa).

For pH, no differences were observed between treatments, time, or their interaction ($P \geq 0.05$; Table 4). This coincides with what was previously reported[34,35,36], where they evaluated the effect of moringa leaf extracts on different fermentation kinetics and observed no differences between treatments. These results guarantee the viability of the rumen microbiota[37]. On the other hand, for dry matter (DM) digestibility, there were differences between treatments ($P<0.05$); it was higher for the HM treatment (30 % moringa). This differs from what was reported by other researchers[38], who found a decrease in digestibility as the concentration of moringa in feed rations increases; nevertheless, it coincides with Morsy *et al*[6], who observed that, when the proportion of moringa extract increases, digestibility increases. This increase in digestibility is related to changes in the amount of NDF and ADF, which decreased as the level of moringa in the diet increased (Table 3), allowing to observe the impact that the addition of this ingredient has on a whole diet. This difference in dry matter digestibility may be related to differences in the composition of bacterial communities[6], which could have been impacted by the presence and increase of moringa in the diet. Gas production (GP) showed differences between treatments and was higher for HM treatment ($P<0.05$; Table 4); however, this increase did not have the desired impact on the end products of fermentation, where the total volatile fatty acids (TVFAs) were lower for the HM treatment (Figure 1). This is directly related to the production of CO_2 (Figure 2), which showed the same behavior as the TVFAs, decreasing as the level of moringa in the diet increased ($P<0.05$). On the other hand, methane (CH_4) production did not show the same behavior; it can be observed that the LM treatment was lower than the other treatments ($P<0.05$, Figure 3). Finally, for NH_3, there were no differences between treatments, time, or their interaction ($P>0.05$; Table 4).

Figure 1: Total production of volatile fatty acids

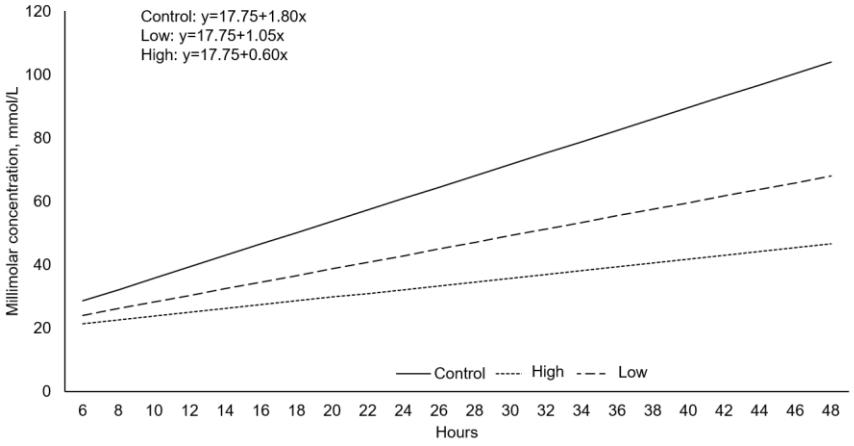


Figure 2: CO_2 production

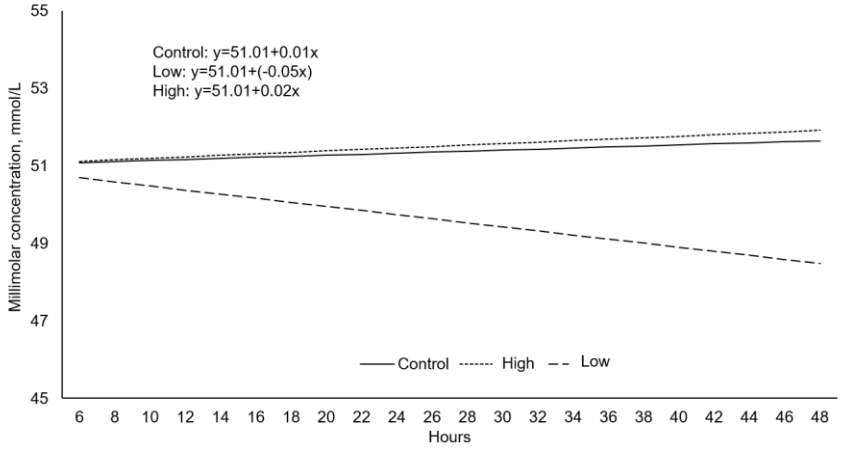
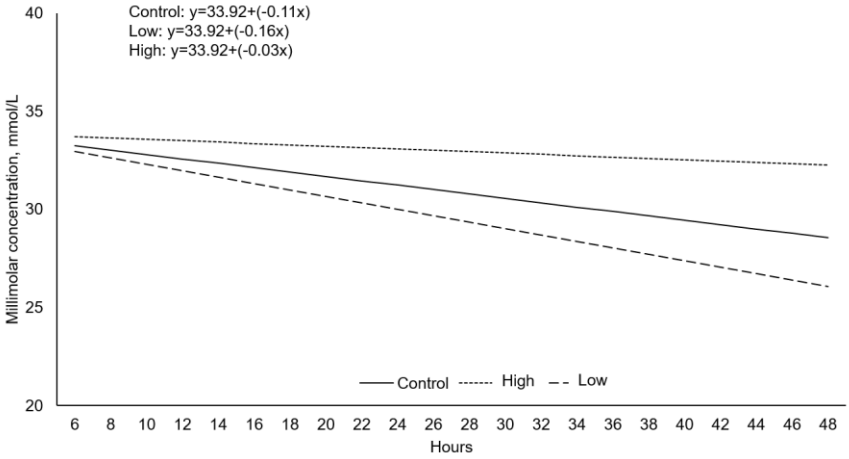


Figure 3: CH_4 production



CO_2 and CH_4 emissions during rumen fermentation cause an energy loss of between 2 and 12 %[36]. The reduction of methane due to the presence of *Moringa oleifera* has also been reported in other studies[24,35,39]. It is also interesting to note that, at higher levels of moringa, methane concentrations increase. It is well documented that secondary metabolites of some plant species can mitigate CH_4 production in rumen fermentation and *Moringa oleifera* is rich in secondary metabolites, such as tannins, saponins, and other phenolic compounds that have antimicrobial and antiprotozoal properties that, consequently, could be modifying the composition of the microbiota and thus the production of CH_4[6].

The molar percentage of the millimolar concentrations of the VFAs was also calculated for the three treatments (Table 5). The fermentation pattern of the three diets was mainly acetic, since this acid was the one that was found in greater proportion. This coincides with what has been reported in the literature, where it is mentioned that, in forage-based diets, the concentration of acetic acid is usually between 60 and 75 %[40]. Regarding propionic acid, the literature indicates that its proportion in this type of diet varies between 15 % and 19 %[40]. Nonetheless, a higher proportion was obtained in this experiment, with the lowest being 23.82 % in the HM treatment and the highest being 30.93 % in LM. The proportion of butyric acid also coincides with that reported in the literature[40]; however, it is at the lowest levels of the expected range, which varies between 8 and 16 %.

Table 5: Comparison of the concentration of volatile fatty acids (VFAs) in mmol/L and their conversion to molar percentage

	CA		**LM**		**HM**	
	mmol/L	**%**	**mmol/L**	**%**	**mmol/L**	**%**
Total VFA	103.9	100	67.9	100	46.6	100
Acetic acid	63.9	61.5	42.3	62.3	31.2	66.95
Propionic acid	30.6	29.45	21	30.93	11.1	23.82
Butyric acid	9.3	8.95	4.6	6.77	4.2	9.01

CA= control treatment (100 % alfalfa); LM= low moringa treatment (15 % moringa 85 % alfalfa); HM= high moringa treatment (30 % moringa 70 % alfalfa).

Conclusions and implications

Almost all fermentation parameters were affected by the presence of *Moringa oleifera*, except for pH and N-NH_3. Although the obtained concentrations of NDF, ADF, and TVFA were not as expected, they are in acceptable proportions for ruminant feed. Nevertheless, some of the variables had desirable behaviors, such as dry matter digestibility, production of gas, carbon dioxide and methane. It should be noted that both CO_2 and CH_4 had a decrease

in the treatment of moringa at 15 % (LM), which could be due to the presence of the secondary metabolites of this plant, which, in turn, affect the population of protozoa and methanogenic microorganisms. Similarly, increasing the amount of moringa to 30 % may also be increasing the antinutritional factors of *M. oleifera*, which can affect the rumen microbiota. Due to the above, it is proposed to perform rumen population analyses to identify the microorganisms present and the active metabolic pathways to find their relationship with these results.

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Conflicts of interest

All authors declare that there are no conflicts of interest of any kind in the publication of this document.

Literature cited:

1. Su B, Chen X. Current status and potential of *Moringa oleifera* leaf as an alternative protein source for animal feeds. Front Vet Sci 2020;7:53 https://doi.org/10.3389/fvets.2020.00053.

2. Quintanilla-Medina J, Joaquín-Cancino S, Martínez-González J, Limas-Martínez A, López-Aguirre D, Estrada-Drouaillet B, Hernández-Meléndez J. Usos de *Moringa oleifera* Lam. (Moringaceae) en la alimentación de rumiantes. Agroproductividad 2018;11:89-93.

3. Grossi G, Goglio P, Vitali A, Williams A. Livestock and climate change: impact of livestock on climate and mitigation strategies, Anim Front 2019;9(1):69–76 https://doi.org/10.1093/af/vfy034.

4. Avila-Serrano NY, López-Garrido SJ, Galicia-Jiménez MM, González-Crespo GJ, Camacho-Escobar MA. Efecto de la incorporación de arbóreas a dietas de *Cynodon nlemfuensis* durante la fermentación ruminal *in vitro.* Terra Latinoam 2020;38(2):403-412.

5. Sánchez-Machado DI, Núñez-Gastélum JA, Reyes-Moreno C, Ramírez-Wong B, López-Cervantes J. Nutritional quality of edible parts of *Moringa oleifera*. Food Anal Methods 2010;3:175–180. https://doi.org/10.1007/s12161-009-9106-z.

6. Morsy TA, Gouda GA, Kholif AE. *In vitro* fermentation and production of methane and carbon dioxide from rations containing *Moringa oleifera* leave silage as a replacement of soybean meal: *in vitro* assessment. Environ Sci Pollut Res 2022;29:69743–69752. https://doi.org/10.1007/s11356-022-20622-2.

7. Babiker EE, Juhaimi FAL, Ghafoor K, Abdoun KA. Comparative study on feeding value of *Moringa* leaves as a partial replacement for alfalfa hay in ewes and goats. Livest Sci 2017;195:21-26.

8. Kashyap P, Kumar S, Riar CS, Jindal N, Baniwal P, Guiné RPF, Correia PMR, Mehra R, Kumar H. Recent advances in drumstick (*Moringa oleifera*) leaves bioactive compounds: composition, health benefits, bioaccessibility, and dietary applications. Antioxidants 2022;11-402. https://doi.org/10.3390/antiox11020402.

9. Premi M, Sharma HK. Effect of extraction conditions on the bioactive compounds from *Moringa oleifera* (*PKM 1*) seeds and their identification using LC–MS. Food Meas 2017;11:213–225. https://doi.org/10.1007/s11694-016-9388-y.

10. Teixeira EMB, Barbieri-Carvalho MR, Neves VA, Silva MA, Arantes-Pereira L. Chemical characteristics and fractionation of proteins from *Moringa oleifera* Lam. leaves. Food Chem 2014;147:51-54.

11. Seradj AR, Morazan H, Fondevila M, Liang JB, De la Fuente G, Balcells J. *In vitro* and *in situ* degradation characteristics and rumen fermentation products of *Moringa oleifera* harvested at three different ages. Trop Anim Sci J 2019;42(1):39-45. https://doi.org/10.5398/tasj.2019.42.1.39.

12. Babiker EE, Juhaimi FAL, Ghafoor K, Mohamed HE, Abdoun KA. Effect of partial replacement of alfalfa hay with *Moringa* species leaves on milk yield and composition of Najdi ewes. Trop Anim Health Prod 2016;48:1427-1433.

13. Khan I, Zaneb H, Masood S, Yousaf MS, Rehman HF, Rehman H. Effect of *Moringa oleífera* leaf powder supplementation on growth performance and intestinal morphology in broiler chickens. J Anim Physiol Anim Nutr 2017;101(1):114-121. doi: 10.1111/jpn.12634.

14. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74:3583–3597.

15. AOAC. Official methods of analysis. 16th ed. AOAC International, Gaithersburg, MD: Association of Official Analytical Chemists. 1997.

16. Theodorou MK, Williams BA, Dhanoa MS, McAllan AB. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Tech 1994;48:185-197.

17. Menke KH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Anim Res Dev 1988;28:7–55.

18. Ankom Technologies. *In Vitro* True Digestibility using the ANKOM DAISYII Incubator. 2023. https://www.ankom.com/sites/default/files/document-files/Method_3_InVitro_D200_D200I.pdf

19. Broderick GA, Kang JH. Automated simultaneous determination of ammonia and total amino acids in ruminal fluids and *in vitro* media. J Dairy Sci 1980;63:64-75.

20. Galyean ML. Laboratory procedures in animal nutrition research. Department on Animal and Food Sciences. Texas Tech University, Lubbock, TX, USA. 1980:161-162.

21. Wolin M J. A theoretical rumen fermentation balance. J Dairy Sci 1960;42:1452-1459.

22. SAS. SAS/STAT User's Guide: Statics (version 9.1). Cary, North Carolina, USA. 2004.

23. Sultana S. Nutritional and functional properties of *Moringa oleifera*. Metabol Open 2020;9(8):100061. doi: 10.1016/j.metop.2020.100061.

24. Soliva C, Kreuzer M, Foidl N, Foidl G, Machmueller A, Hess H. Feeding value of whole and extracted *Moringa olifera* leaves for ruminants and their effects on ruminal fermentation *in vitro*. Anim Feed Sci Tech 2005;118:47-62 https://doi.org/10.1016/j.anifeedsci.2004.10.005.

25. Dhakar RC, Maurya S, Pooniya B, Bairwa N, Gupta M, Sanwarmal. Moringa: The herbal gold to combat malnutrition. Chron Young Sci 2011;2(3):119-125.

26. Kholif AE, Gouda GA, Morsy TA, Salem AZM, Lopez S, Kholif AM. *Moringa oleifera* leaf meal as a protein source in lactating goat's diets: Feed intake, digestibility, ruminal fermentation, milk yield and composition, and its fatty acids profile Small Rumin Res 2015;129:129-137. https://doi.org/10.1016/j.smallrumres.2015.05.007.

27. Olusanya RN, Kolanisi U, Van-Onselen A, Ngobese NZ, Siwela M. Nutritional composition and consumer acceptability of *Moringa oleifera* leaf powder (MOLP)-supplemented mahewu. S Afr J Bot 2020;129:175-180. https://doi.org/10.1016/j.sajb.2019.04.022.

28. Luo C, Donghai W, Na L, Haiqing L, Gaofei L, Zhijun C, *et al*. Analysis of chemical composition, amino acid content, and rumen degradation characteristics of six organic feeds. Animals 2022;12(6):682. https://doi.org/10.3390/ani12060682.

29. Okiki PA, Osibote IA, Balogun O, Oyinloye BE, Idris OO, Adelegan O, Olagbemide PT. Evaluation of proximate, minerals, vitamins and phytochemical composition of *Moringa oleifera* Lam. cultivated in Ado Ekiti, Nigeria. Adv Biol Res 2015;9(6):436-443.

30. Cofré P. Suplementación de calcio y fósforo a vacas en lactancia. Instituto Nacional de Investigaciones Agropecuarias. 1986. https://biblioteca.inia.cl/items/a7de0e93-9c21-457a-b356-e23d58425163.

31. Wild KJ, Siegert W, Windisch WM, Südekum KH, Rodehutscord M. Meta-analysis-based estimates of efficiency of calcium utilization by ruminants. Animal 2021;15(8): 100315. https://doi.org/10.1016/j.animal.2021.100315.

32. Velázquez-Zavala M, Peon-Escalante IE, Zepeda-Bautista R, Jimenez-Arellanes MA. Moringa (*Moringa oleífera* Lam.): potential uses in agriculture, industry and medicine. Rev Chapingo Ser Hort 2016;22(2):95-116.

33. Hassan FU, Guo Y, Li M, Tang Z, Peng L, Liang X, Yang C. Effect of methionine supplementation on rumen microbiota, fermentation, and amino acid metabolism in *in vitro* cultures containing nitrate. Microorganisms. 2021;9(8):1717. doi: 10.3390/microorganisms9081717. PMID: 34442796; PMCID: PMC8397988.

34. Kholif AE, Gouda GA, Morsy TA, Matloup OH, Sallam SM, Patra AK. Associative effects between *Chlorella vulgaris* microalgae and *Moringa oleifera* leaf silage used at different levels decreased in vitro ruminal greenhouse gas production and altered ruminal fermentation. Environ Sci Pollut Res 2023;30:6001–6020. https://doi.org/10.1007/s11356-022-22559-y.

35. Parra-Garcia A, Elghandour MMMY, Greiner R, Barbabosa-Pliego A, Camacho-Diaz LM, Salem AZM. Effects of *Moringa oleifera* leaf extract on ruminal methane and carbon dioxide production and fermentation kinetics in a steer model. Environ Sci Pollut Res Int 2019;26(15):15333-15344. doi: 10.1007/s11356-019-04963-z.

36. Pedraza-Hernández J, Elghandour MMMY, Khusro A, Camacho-Diaz LM, Vallejo LH, Barbabosa-Pliego A, Salem AZM. Mitigation of ruminal biogases production from goats using *Moringa oleifera* extract and live yeast culture for a cleaner agriculture environment. J Clean Prod 2019;234:779-786. https://doi.org/10.1016/j.jclepro.2019.06.126.

37. Cerrato M, Calsamiglia S, Ferret A. Efectos del tiempo a pH subóptimos y el número de ciclos sobre la fermentación microbiana ruminal en cultivo continuo. ITEA 2005;26(2):578-580.

38. Quintanilla-Medina JJ, López-Aguirre D, Martínez-González JC, Limas-Martínez AG, Lucero-Magaña FA, Ruíz-García S, Hernández-Meléndez J. Digestibilidad *in vitro* de dietas con diferentes niveles de inclusión de moringa (*Moringa oleifera*) para corderos en crecimiento. Rev Investig Vet Perú 2020;31(3):e16840. https://dx.doi.org/10.15381/rivep.v31i3.16840.

39. Dey A, Shyam P, Poonam P. Potential of *Moringa oleifera* leaves in modulating *in vitro* methanogenesis and fermentation of wheat straw in buffalo. Indian J Anim Sci 2014;84:533-538.

40. Zavaleta E. Los ácidos grasos volátiles, fuente de energía en los rumiantes. https://www.fmvz.unam.mx/fmvz/cienciavet/revistas/CVvol1/CVv1c09.pdf.