IMPACT OF SUPPLEMENTARY MORINGA OLEIFERA LEAF EXTRACT ON RUMINAL NUTRIENT DEGRADATION AND MITIGATING METHANE FORMATION IN VITRO

Yosra A. Soltan1*, A. S. Morsy2, Nesreen M. Hashem1, and S. M.A. Sallam1

1 Department of Animal and Fish production, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

2 Livestock Research Department, Arid Land Cultivation Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt.

*Corresponding author. Email: uosra_eng@yahoo.com

(Received 6/2/2019, accepted 27/3/2019)

SUMMARY

Plant extracts may be highly effective as natural dietary supplementation options to alternate the dietary antibiotics as growth promoters in ruminant diets. The current study was conducted to evaluate the dose response effects of the moringa (Moringa oleifera) leaf extract (MLE) as a natural alternative to monensin in sheep diets, on ruminal methane production (CH4), gas production (GP), nutrient degradability and fermentation parameters. The in vitro semi-automatic system of GP was used. The treatments were MLE added to a basal diet (consisted of 50 concentrate: 50 forage) at 0 (control), 50 (MLE low) and 500 (MLE high) mg/kg dry matter, and the ionophore antibiotic monensin was added at 40 mg/kg dry matter. Abundant quantities of essential amino acids, monosaccharides, glycosides and benzene derivatives phytochemicals components were detected by the GC–MS analysis of MLE. The most effective treatments to decrease (P < 0.05) CH4 were monensin and MLE high, while only MLE high enhanced (P < 0.05) the overall mean of total volatile fatty acids (VFAs) concentrations compared to the other treatments and the molar proportion of acetate compared to monensin. A decline (P < 0.05) in protozoal count was observed by monensin, while such effect did not appear at other treatments. No significant differences were observed among the experimental treatments in the ruminal degradability, ammonia concentrations or GP. This study demonstrated efficiency of MLE as an effective natural intervention to monensin in sheep diets.

Keywords: Methanogenesis, monensin, ruminal fermentation and moringa leaf.

INTRODUCTION

Methane (CH4) emission from ruminants represents a considerable loss of dietary energy which could potentially be redirected towards the meat or milk production (Patra and Yu, 2015). This is especially the case in most areas of the developing countries, due to the low feed efficiency of the animals that lead to high cost in terms of CH4 produced per unit of animal product (Soltan et al., 2012).

Antibiotic ionophores are widely used in ruminant industry to improve energy and protein utilization and decrease CH4 emission (Russell and Strobel, 1989). However, there is a controversy about the use of these additives due to the risk of transferring residues into final animal products (meat and/or milk). These concerns have promoted the search for transferring natural additives such the secondary metabolites which occurring naturally in many plant species.

Moringa trees (Moringa oleifera) belonging to the Moringaceae family, have been used as an antibiotic in traditional medicine dates back thousands of years in many developing countries (Soliva et al., 2005 and Soltan et al., 2018). Among moringa parts, the leaf was the most part rich in various phytochemicals with high potency as antimicrobial, anti-cancerous, antianthelmintic, antispasmodic, anti-inflammatory properties (Sholapur and Patil 2013; Wang et al., 2016 and Soltan et al., 2017a). Thus there are many studies confirmed moringa leaves as dietary feed additives in livestock production, however, most of these studies were done for the whole leaf, while eliminating its extractions. Moreover, most of studies with the leaf extracts eliminated their antimethanogenic activity. Parts other than the leaves of moringa were suggested to
be natural alternatives to monensin antibiotic to modulate CH\textsubscript{4} emission towards more volatile fatty acids. Recently, Soltan \textit{et al.} (2018), found a similarity between moringa whole root bark and monensin in enhancing the growth performance of the growing lambs, while reduced CH\textsubscript{4} emission relative to body weight gain. Thus, this provided a suggestion that if the chemical characterization of secondary metabolites of moringa leaf extract (MLE) and their mechanism of action can be clarified, they may introduce an alternative to replace the dietary antibiotic additives for ruminants. The objective of the current study is to evaluate \textit{in vitro} the effects of two levels of MLE on ruminal fermentation, degradability and CH\textsubscript{4} production compared to monensin.

**MATERIALS AND METHODS**

\textit{Moringa origin, processing and analysis of the MLE:}

Fodder leaves of Moringa \textit{(Moringa oleifera)} had been harvested in the first cutting. About 25 kg of fresh leaves were collected from a private farm located 45 km south of Alexandria (30°50′56″N 29°36′42″E), Egypt. The leaves were collected from 50 trees, pooled, dried at 40°C for 72 h and milled through 1 mm screen.

Ten grams of moringa leaves were ground to a fine powder and mixed with 100 ml ethanol (700 ml/l). The mixture was then ultrasonically for 30 min. The ethanol extract solution was subsequently filtered and kept at −5 °C overnight and was filtered again. The supernatant was transferred to the rotary evaporator (RE301/601/801, Yamato Scientific America Inc., USA) and treated at 42 °C for 30 min in order to remove the ethanol. The concentrated extract recovered in the volumetric flask was lyophilized for 3 days to get the experimental MLE that was used for the chemical analysis and the \textit{in vitro} assay. The MLE was subjected to an in-depth compositional analysis using gas chromatography/mass spectrometry (Thermo Scientific TRACE- 1300 series GC) as described in details by Soltan \textit{et al.} (2018).

\textit{Basal diet, treatments and inocula preparation:}

The control total mixed ration was consisted of (g/kg DM): 500 g clover \textit{(Trifolium alexandrinum)} hay, 200 g ground maize, 27.5 g soybean meal, 125 g cotton seed meal, 20 g limestone, 10 g sodium chloride and 3 g mineral premix. The ration was chemically analyzed based on DM (g/kg) according to AOAC (1995) as: OM= 896.7and CP= 141.6 (as 6.25×N). The neutral detergent fiber (NDF) =505.9, acid detergent fiber (ADF) = 252.6, and lignin= 41 were measured sequentially using ANKOM Technology Corporation, Macedon, NY, USA, and expressed exclusive of residual ash as described by Goering and Van Soest (1970) and Van Soest (1973). The diet was formulated to meet NRC (2007) nutrient requirements recommended for growing sheep.

Four experimental treatments were evaluated as follow: control (the basal diet without supplementations, monensin [(the basal diet supplemented with the manufacturer’s recommendation dose (40 mg/kg DM) of ionophore sodium monensin (Rumensin®, Elanco, Itapira, Brazil)], MLE was supplemented to the basal diet at two doses 50 (MLE\textsubscript{low}) or 500 (MLE\textsubscript{high}) mg/ kg DM, respectively. The ionophore antibiotic monensin was selected because it is among the most common additives used to decrease CH\textsubscript{4} emission and modulate ruminal fermentation characters (Soltan \textit{et al.}, 2018).

Four adult rumen-cannulated Barki sheep (58± 2.5 kg body weight) were used as inoculum donors. The donor animals were fed \textit{ad libitum} berseem clover hay and a concentrate feed mixture (0.7 kg/100 kg body weight, and containing 145 g/kg DM crude protein), and had free access to fresh water. Each treatment was incubated in four inocula, with each inoculum, four bottles per treatment were prepared, two for truly degraded organic matter (TDOM) determination and the other two for estimating the fermentation parameters. The same procedure was applied for the blanks (bottles containing the ruminal inoculum and the buffer solution without samples) to be able to correct the GP from the inoculum, and for an internal standard (bottles containing clover hay, ruminal inoculum and the buffer solution) to correct for sensitivity changes induced by the inoculum (Soltan \textit{et al.}, 2012).

\textit{In vitro assay:}

A semi-automatic system of GP (Bueno \textit{et al.}, 2005) using a pressure transducer and a data logger (GN200, Sao Paulo, Brazil) with some modifications according to Soltan \textit{et al.} (2018) was used.
For CH₄ determination, 2 ml of the head space gas was sampled by a syringe (med Dawliaico, Assiut, Egypt) at each measuring event and stored in a 10 ml vacutainer tubes (BD Vacutainer® Tubes, NJ, USA). Methane concentration was determined using a gas chromatograph (Model 7890, Agilent Technologies, Inc, Colorado 80537, USA), the separation conditions in details were described by Soltan et al. (2018). The test of linearity and calibration were accomplished using a standard gas curve in the range of probable concentrations of the samples using pure CH₄ (Abu Qir Petroleum Co., Alexandria, Egypt; 939 ml/l purity). The amounts of CH₄ produced were calculated according to Longo et al. (2006).

After termination of the incubation, all bottles were placed in ice to inhibit fermentation. Two bottles were assigned to the determination of the truly degraded dry matter and organic matter (TDDM and TDOM, respectively) following Blümmel and Becker (1997) method. The partitioning factor (PF; an indicator of ruminal microbial syntheses) was calculated as the ratio of TDOM (mg) and gas volume (ml) (Blümmel et al., 1997). The incubation liquid of the other two bottles was used for determining fermentation parameters and protozoal counts. The ammonia concentrations were evaluated calorimetrically by spectrophotometer (Alpha-1101 model; Labnics Equipment, California, USA) using commercial lab test (Konitzer and Voigt, 1963). The VFAs were determined following the method of Palmquist and Conrad (1971) using a gas chromatograph (Thermo fisher scientific, Inc., TRACE1300, Rodano, Milan, Italy) with some modifications described in details by Soltan et al. (2018). Protozoal abundance was counted by microscopy following the procedure of Dehory et al. (1983).

Statistical analysis:

Data were subjected to analysis of variance (ANOVA), using the PROC MIXED of SAS software package (2002). The four inocula were considered as the true statistical replicates. Each treatment was incubated in duplicate (analytical replicates) to achieve highly accurate estimate of a true replicate. The analytical replicates were averaged prior to statistical analysis with each inoculum being the statistical replicate, thus the statistical number of replications of treatments (n = 4) are the true statistical replications. The significant differences between individual means were considered significant at P < 0.05, whereas 0.05 < P < 0.10 were considered as a tendency by using Tukey test.

RESULTS AND DISCUSSION

GC–MS analysis of MLE:

Under the current GC–MS separation conditions, the most abundant compounds identified for MLE were branched chain amino acids (BCAA) since L-valine, L-alanine, L-leucine and L-isoleucine were 4.14, 3.90, 2.72 and 2.65%, respectively. Other amino acids like L-threonine (1.38%) was detected, components with benzene ring were detected in high concentration, glycosides were found (7.3%), also 3-caffeoylquinic acid was 3.95% and butanedioic acid was 4.92% (Table 1). These combinations of moringa active components are found to be nutritionally and biologically active, e.g. its bioactive benzene fraction and glycosides are known to possess antibacterial, antifungal, and antioxidant properties (Alptüzün et al., 2006). Moreover, MLE can be considered as a good source of amino acids, because it contains considerable amounts of BCAA. These results are in accordance with Gopalakrishnan et al. (2016) who suggested that moringa leaves can be considered as a protein supplement due to the high content of essential amino acids (440 mg/kg DM). Although MLE have high content of various active components, little information is available about the effects of these components on ruminal fermentation or methanogenesis.

Ruminal CH₄ fermentation parameters and degradability:

The results presented in Table (2) showed that no differences were observed for the gas production (GP), truly degraded dry matter (TDDM), truly degraded organic matter (TDOM) and partitioning factor (PF) among all the experimental treatments. The most efficient treatments to decrease (P < 0.05) CH₄ were monensin and MLE high, where their proportional CH₄ reduction was 18.1 and 15.5%, respectively compared to the control. Currently, MLE high seemed to act against ruminal methanogenesis, thus likely adversely affecting Archaea as monensin did, and this finding suggest that MLE can be an alternative to the critical antibiotics feed additives in ruminant diets without adverse effects on GP or ruminal degradability. Previous studies also confirmed the antimethanogenic activity of moringa leaves studies, e.g. Dey et al. (2014) found an achievement of CH₄ inhibition combined with enhancement of the total GP and organic matter.
degradability by wheat straw supplemented with moringa leaves in buffalo diets. Soltan et al. (2014) reported that extracts of moringa leaves and root barks could be used as effective natural alternatives to monensin in sheep diets, not only to decrease CH4 emission, but also to increase the ruminal nutrient degradability. Similarly, Soliva et al. (2005) found that CH4 production was inhibited significantly by 17% with moringa leaves based diet as compared to the diets containing rapeseed meal or soybean meal, without adverse effects on the ruminal fermentation or nutrient degradability, the authors suggested that such effects might relate to existence of bioactive components in moringa leaves, however no specific bioactive components were assigned to confirm such suggestion.

Table (1): Individuality of constituents in the moringa leaf extract (MLE) determined by gas chromatography/mass spectrometry.

| Peak | Name | % Area | RT |
|------|------|--------|----|
| 1    | Benzene, 1,1'-[4-(3-phenylpropyl)-1,7-heptanediyl]bis-(CAS) | 8.17 | 2.03 |
| 2    | L-alanine, N-(trimethylsilyl)-, trimethylsilyl ester | 3.90 | 7.295 |
| 3    | L-valine, N-(trimethylsilyl)-, trimethylsilyl ester | 4.14 | 10.090 |
| 4    | L-leucine, N-(trimethylsilyl)-, trimethylsilyl ester | 2.72 | 11.519 |
| 5    | L-isoleucine, N-(trimethylsilyl)-, trimethylsilyl ester | 2.65 | 12.085 |
| 6    | Hexasiloxane, 1,1,3,3,5,7,7,9,11,11-dodecamethyl- | 2.88 | 12.147 |
| 7    | Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester | 0.77 | 13.097 |
| 8    | Carotene, 3,4-didehydro-1,1',2,2'-tetrahydro-1'-hydroxy-1-methoxy- | 3.03 | 13.842 |
| 9    | L-threonine, N-Obis[(trimethylsilyl)], trimethylsilyl ester | 1.38 | 14.533 |
| 10   | Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester | 4.92 | 17.032 |
| 11   | Nd | 2.40 | 17.763 |
| 12   | Trimethylsil 2,3,4-tris[(trimethylsilyl)oxy]butanone | 2.54 | 18.896 |
| 13   | Glucuronic acid, methyl 2,3,5,6-tetakis-o-(trimethylsilyl)-, .alpha.-D- | 1.61 | 24.118 |
| 14   | D-fructose, 1,3,4,5,6-pentakis-o-(trimethylsilyl)- | 6.02 | 24.335 |
| 15   | D-fructose, 1,3,4,5,6-pentakis-o-(trimethylsilyl)- | 4.73 | 24.494 |
| 16   | Mannofuransio, methyl 2,3,5,6-tetakis-o-(trimethylsilyl)-, .alpha.-D- | 3.57 | 25.105 |
| 17   | no-2,4-dimethyl-1H-pyrrol-3-y]-2-methyl-4H-pyran-3-carboxylic acid ethyl ester | 1.95 | 25.464 |
| 18   | Beta-D-galactofuranose, 1,2,3,5,6-pentakis-o-(trimethylsilyl)- | 6.90 | 27.940 |
| 19   | Beta.-D-galactofuranose, 1,2,3,5,6-pentakis-o-(trimethylsilyl)- | 2.69 | 28.229 |
| 20   | 4-(Pentadeuterio)phenylazulene | 1.85 | 29.849 |
| 21   | Colchicine | 1.86 | 34.958 |
| 22   | Benzene, 2-(1-decyl-1-undecenyl)-1,4-dimethyl- (CAS) | 9.15 | 37.203 |
| 23   | 2,3-Bis(3'-methoxy-2'-nitro phenylimino)-2H-indole | 2.40 | 37.547 |
| 24   | 1,3,4,6-tetakis-o-(trimethylsilyl)hex-2-ulosufuranosyl 2,3,4,6-tetakis-o- (trimethylsilyl)hexopyranoside | 7.30 | 38.176 |
| 25   | Alpha.-D-glucopyranoside, 1,3,4,6-tetakis-o-(trimethylsilyl)-beta.-d fructofuranosyl 2,3,4,6-tetakis-o-(trimethylsilyl)- | 5.96 | 38.973 |
| 26   | 2(3,4is[(trimethylsilyl)oxy]phenyl) - 3,5,7 tris[(trimethylsilyl)oxy]-4-hchromen-4-one | 0.56 | 45.744 |
| 27   | Hexa(trimethylsilyl)trans-3-o-caffeoyl-d-quinic acid | 3.95 | 46.101 |

Nd: not detected

Table (2): Effect of monensin, and moringa leaf extract (MLE) on ruminal gas production (GP), degradability and partitioning factor (PF).

| Item          | Control | Monensin | MLE_Low | MLE_High | SEM     | P value |
|--------------|---------|----------|--------|----------|---------|--------|
| GP (mL/g DM) | 150.4   | 143.3    | 160.4  | 159.6    | 22.71   | 0.4241 |
| CH4 (mL/L TDOM) | 34.80a | 28.49b   | 32.95a | 29.39b   | 7.861   | 0.0199 |
| TDDM (g/kg)  | 620.5   | 611.3    | 603.4  | 610.8    | 41.189  | 0.1214 |
| TDOM (g/kg)  | 597.06  | 595.8    | 562.7  | 610.9    | 38.344  | 0.1993 |
| Partitioning factor (PF) | 2.2010 | 1.9396   | 1.939  | 2.431    | 0.4148  | 0.216  |

GP: net gas production; CH4: methane; TDDM: truly degraded dry matter; TDOM: truly degraded organic matter; SEM: Standard error of the mean.

ab: Means within a row without a common superscript letter differ significantly (P < 0.05).

Most common methanogen inhibitors negatively affect the ruminal fermentation or/ and organic matter degradability at doses that achieve desirable methane reduction (Patra and Yu, 2015). Currently, although
the reasons for methane inhibition caused by MLE remain to be explored, it seems that MLE affected the methanogenesis directly, since the ruminal degradability and the protozoal counts remained unchanged. Thus combinations between antibacterial and antioxidant bioactive components in MLE might play a key role in that concern (Alptüzün et al., 2009; Soltan et al., 2018).

Table (3) presented the in vitro effects of MLE and monensin on ruminal pH, ammonia concentrations, protozoal count and VFAs. No significant differences were observed among the experimental treatments in the ruminal pH or ammonia concentrations, while a decline (P < 0.05) in protozoal count was observed by monensin, but such effect did not appear at other treatments. Methanogen inhibitors can reduce CH₄ production directly or indirectly ways through the inhibition of numbers or activity of methanogens and antiprotozoal properties, respectively (Cieslak et al., 2013). Monensin was found to alternate the ruminal hydrogen-sink products directly towards less CH₄ production through a shift in hydrogen usage from methanogenesis and/or formate to propionate or succinate production by the inhibition of gram-positive bacteria (Russell and Strobel, 1989 and Schären et al., 2017). Moreover, the antiprotozoal effect of monensin might partially help to explain the indirect reduction in CH₄ emission. Thus currently, the tendency (P = 0.08) in enhancement of propionate production and decreasing (P < 0.05) the acetate to propionate ratio caused by monensin without affecting the total VFAs production may support the above suggestion. On other hand, the reduction of CH₄ caused by MLE_high was combined by an enhancement (P < 0.05) in the total VFAs production. This may be due to the presence of components with antioxidant activity (e.g. glycosides and 3-caffeoylquinic acid) in MLE. Recently, many studies confirmed that the presence of these components would lessen oxidative stress and promote better conditions for ruminal fermentation (Soltan et al., 2017b, 2018). Such enhancement in total VFAs production found by MLE_high might partly confirm this hypothesis, since VFAs are the principal outcome of the ruminal fermentation (Calsamiglia et al., 2007). The reasons for the increases in VFAs by MLE are not clear, however the high content of essential amino acids (BCAA) found in MLE may parley explain such effect. Nouman et al. (2014) reported that the dietary supplementation of valine, leucine and isoleucine enhanced the production of total VFAs. Thus, the current results suggested that the fermentation pathways expended H₂ to produce VFAs than CH₄ and these increases of VFAs could be attributed to acetate enhancement, hence acetate are the major part of the total VFAs produced by ruminal microbes (Calsamiglia et al., 2007 and Soltan et al., 2017b). Reduction of CH₄ combined with enhancement (P < 0.05) of acetate caused by MLE_high may suggest that MLE stimulates acetogenesis as an alternative to the ruminal methanogenesis. Ruminal methanogenesis pathway is the primary H₂ sink, while acetogens have a poorer affinity to H₂ than methanogens (Tan et al., 2011). Thus the current results may refer to a competition happened between methanogenesis and acetogenesis for H₂ binding. Recently, many studies have also suggested that acetogenesis can serve as an alternative hydrogenotrophic pathway in the rumen (El-Zaïat et al., 2014 and Soltan et al., 2017b).

Table (3): Effect of monensin and moringa leaf extract (MLE) on some ruminal parameters.

| Item          | Control | Monensin | MLE_Low | MLE_High | SEM   | P value |
|---------------|---------|----------|---------|----------|-------|---------|
| pH            | 5.98    | 5.99     | 5.93    | 5.94     | 0.327 | 0.991   |
| NH₃-N (mg/100 mL) | 22.1    | 22.8     | 24.5    | 24.7     | 4.557 | 0.110   |
| VFAs          |         |          |         |          | SEM   | P value |
| Total (mM)    | 46.9ᵇ   | 46.7ᵇ    | 48.1ᵇ   | 57.9ᵇ    | 1.267 | 0.001   |
| Acetate, %    | 64.2ᵃᵇ  | 61.4ᵇ    | 63.7ᵃᵇ  | 64.8ᵇ    | 0.687 | 0.028   |
| Propionate, % | 16.2     | 18.5     | 16.9    | 16.5     | 0.358 | 0.082   |
| Butyrate, %   | 14.1     | 15.39    | 14.0    | 15.3     | 0.467 | 0.164   |
| Isovalerate, %| 1.07     | 1.18     | 1.09    | 1.18     | 0.120 | 0.880   |
| Valerate, %   | 1.68     | 1.79     | 1.62    | 1.67     | 0.100 | 0.663   |
| Isovalerate, %| 1.56     | 1.80     | 1.57    | 1.78     | 0.246 | 0.889   |
| C2:C3        | 3.74     | 3.38     | 3.82    | 3.99     | 0.091 | 0.084   |
| Protozoa ×10⁶ | 4.94ᵇ    | 3.93ᵃᵇ   | 5.38ᵇ   | 5.21ᵇ    | 0.7502| 0.009   |

SEM: Standard error of the mean.
ᵃᵇ Means within a row without a common superscript letter differ significantly (P < 0.05).
The PF is an indicator of the efficiency of microbial protein synthesis (Blümmel et al., 1997), none of the experimental additives affected the PF (Table 3). The lack of change in PF values is consistent with the rather constant of ammonia concentrations suggested that the nitrogen use by microbes for their protein synthesis remained unchanged, and the VFAs probably enhanced by ruminal microbes which are not involved in the amino acids degradation.

No differences were detected between MLE \textsubscript{low} and the control treatments either in the ruminal degradability or the fermentation parameters. These findings suggest that MLE \textsubscript{low} was an inadequate dose to affect the ruminal microbial ecosystem, thus it is important to choose the effective dose of MLE to be applicable in the ruminant's diets. Generally, the effects of MLE either through reducing CH\textsubscript{4} or enhancing the production of VFAs may be nutritionally advantageous to ruminants, due to the increases in the energy supply to animals consequently enhance the whole animal productivity.

CONCLUSION

The current study suggested that MLE can be used as an effective additive for ruminants' diets in the field of smart agriculture. Both MLE and monensin exhibited a similar antimethanogenic activity without adverse effects on ruminal degradability however; they were different in their mode of action. Monensin reduced CH\textsubscript{4} through enhancing propionate production, while MLE enhanced the acetate production. These results also suggested that the consideration of MLE as a dietary supplementation to modify the ruminal fermentation was dose dependent. Further research should focus on the \textit{in vivo} long-term effects of the dietary MLE to be applicable as one of the climate smart agriculture practices in the developing countries.

REFERENCES

Alptüzün, V., S. Parlar, H. Hüseyin Taşl and E. Erciyas (2009). Synthesis and Antimicrobial Activity of Some Pyridinium Salts. Molecules, 14: 5203-5215

AOAC (1995). Association of Analytical Chemists, Official Methods of Analysis, 18\textsuperscript{th} ed., Gaithersburg, MD, USA.

Blümmel, M. and K. Becker (1997). The degradability characteristics of fifty-four roughages and roughage neutral-detergent fibre as described by in vitro gas production and their relationship to voluntary feed intake. British Journal of Nutrition, 77: 757–786.

Blümmel, M., H. Steingass and K. Becker (1997). The relationship between in vitro gas production, \textit{in vitro} microbial biomass yield and 15N incorporations for the prediction of voluntary feed intake of roughages. British Journal of Nutrition, 77: 911–921.

Bueno, I.C.S., S.L.S. Filho, S.P. Gobbo, H. Louvandini, D.M.S.S. Vitti and A.L. Abdalla (2005). Influence of inoculum source in a gas production method. Animal Feed Science and Technology, 123: 95–105.

Calsamiglia, S., M. Busquet, P.W. Cardozo, L. Castillejos and A. Ferret (2007). Essential oils as modifiers of rumen microbial fermentation. Journal of Dairy Science, 90: 2580–2595.

Cieslak, A., M. Szumacher-Strabel, A. Stochmal and W. Oleszek (2013). Plant components with specific activities against rumen methanogens. Animal 7: 253–265.

Dehority, B.A., W.S. Damrona and J.B. McLaren (1983). Occurrence of the rumen ciliate Oligoiso trichabubali in domestic cattle (Bostaurus). Applied and Environmental Microbiology, 45: 1394–1397.

Dey, A., S.S. Paul, P. Pandey and R. Rathore (2014). Potential of \textit{Moringa oleifera} leaves in modulating in vitro methanogenesis and fermentation of wheat straw in buffalo. Indian Journal of Animal Science, 84: 533–538.

El-Zaiat, H.M., R.C. Araujo, Y.A. Soltan, A.S. Morsy, H. Louvandini, A.V. Pires, H.O. Patino, P.S. Correa and A.L. Abdalla (2014). Encapsulated nitrate and cashew nut shell liquid on blood and rumen
constituents methane emission, and growth performance of lambs. Journal of Animal Science, 92: 2214–2224.

Goering, H.K., and P.J. Van Soest (1970). Forage fibre analysis (apparatus, reagents, procedures and some applications). Agric. Handbook No. 379. US Agricultural Research Service, Washington, DC.

Gopalakrishnan, L., K. Doriyaa and D.S. Kumar (2016). Moringa oleifera: A review on nutritive importance and its medicinal application. Food Science and Human Wellness, 5: 49–56.

Konitzer, K. and S. Voigt (1963). Direct determination of ammonium in blood and tissue extracts by means of the phenol by chlorite reaction. Clinica Chimica Acta, 8: 5–11.

Longo, C., I.C.S. Bueno, E.F. Nozella, P.B. Godoy, S.L.S. Cabral Filho and A.L. Abdalla (2006). The influence of head-space and inoculum dilution on in vitro ruminal methane measurements. In: Soliva, C.R., Takahashi, J., Kreuzer, M. (Eds.), Greenhouse Gases and Animal Agriculture: An Update Int. Congr. Series No. 1293. Elsevier, The Netherlands, pp. 62–65.

Nouman, W., S.M.A. Basra, M.T. Siddiqui, A. Yasmeen, T. Gull and M.A.C. Alcayde (2014). Potential of Moringa oleifera L. as livestock fodder crop: a review. Turkish Journal of Agriculture and Forestry, 38: 1-14.

NRC (2007). National Research Council. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelid. The National Academy of Sciences, Washington, DC, USA.

Palmquist, D. and H. Conrad (1971). Origin of plasma fatty acid in lactating dairy cows fed high fat diets. Journal of Dairy Science, 54: 1025–1031.

Patra, A.K. and K. Yu (2015). Effects of adaptation of in vitro rumen culture to garlic oil, nitrate, and saponin and their combinations on methanogenesis, fermentation, and abundances and diversity of microbial populations. Frontiers in Microbiology, 119: 127-138.

Russell, J.B. and H.J. Strobel (1989). Effect of ionophores on ruminal fermentation. Mini review. Applied and Environmental Microbiology, 55: 1–6.

SAS (2002). Statistical Analysis System. SAS PC Windows Version 9.2.0. SAS Institute Inc., Cary, NC, USA.

Schären, M., C. Drong, K. Kiri, S. Riede, M. Gardener, U. Meyer, J. Hummel, T. Urich, G. Breves and S. Dänicke (2017). Differential effects of monensin and a blend of essential oils on rumen microbiota composition of transition dairy cows. Journal of Dairy Science, 100: 2765–2783.

Shah, S.K., D.N. Jhade and R. Chouksey (2016). Moringa oleifera Lam. a study of ethnotbotany, nutrients and pharmacological profile. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 7: 2158–2165.

Sholapur, H.P.N. and B.M. Patil (2013). Pharmacognostic and phytochemical investigations on the bark of Moringa oleifera Lam. Indian Journal of Natural Products and Resources, 1: 96–101.

Soliva, C.R., M. Kreuzer, N. Foid, G. Foid, A. Machmüller and H.D. Hess (2005). Feeding value of whole and extracted Moringa oleifera leaves for ruminants and their effects on ruminal fermentation in vitro. Animal Feed Science and Technology, 118: 47–62.

Soltan, Y.A., R.C. Lucas, A.S. Morsy, H. Louvandini and A.L. Abdalla (2014). The potential of Moringa oleifera leaves, root bark and propolis extracts for manipulating rumen fermentation and methanogenesis in vitro. International Symposium on Food Safety and Quality: Applications of Nuclear and Related Techniques IAEA Headquarters, Vienna, Austria, 10–13 November 2014.

Soltan Y.A., A.S. Morsy, N.M. Hashem and S.M. Sallam (2017a). Utilization of Moringa oleifera in ruminant nutrition (Review article). Sustainable Development of Livestock’s Production Systems” (SDLPS)” from 7-9 November, 2017. Department of Animal Production, Faculty of Agriculture, Alexandria University, Egypt.

Soltan, Y.A., A.S. Morsy, R.C. Lucas and A.L. Abdalla (2017b). Potential of mimosine of Leucaena leucocephala for modulating ruminal nutrient degradability and methanogenesis. Animal Feed Science and Technology, 223: 30–41.
Soltan, Y.A., A.S. Morsy, S.M.A. Sallam, H. Louvandini and A.L. Abdalla (2012). Comparative in vitro evaluation of forage legumes (prosopis, acacia, atriplex, and leucaena) on ruminal fermentation and methanogenesis. Journal of Animal Feed and Sciences, 21: 759–772.

Soltan, Y.A., N.M. Hashem, A.S. Morsy, K. M. El-Azrak, A. Nour El-Din and S.M. Sallam (2018). Comparative effects of Moringa oleifera root bark and monensin supplementations on ruminal fermentation, nutrient digestibility and growth performance of growing lambs. Animal Feed Science and Technology, 235: 189–201.

Tan, H.Y., C.C. Sieo, N. Abdullah, J.B. Liang, X.D. Huang and Y.W. Ho (2011). Effects of condensed tannins from Leucaena on methane production, rumenfermentation and populations of methanogens and protezoa in vitro. Animal Feed Science and Technology, 169: 185–193.

Van Soest, P.J. (1973). Collaborative study of acid detergent fibre and lignin. Journal of the Association of Official Analytical Chemists, 56: 781–784.

Wang, L., X. Chen and A. Wu (2016). Mini review on antimicrobial activity and bioactive compounds of Moringa oleifera. Journal of Medicinal Chemistry, 6: 578-582.