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# POTENTIAL OF GRADED DOSES OF NEEM (*AZADIRACHTA INDICA*) SEED OIL ON RUMINAL FERMENTATION CHARACTERISTICS, DEGRADABILITY, AND METHANE FORMATION *IN VITRO*

Waleed Al-Marzooqi[1], Sobhy M. Sallam[2], Othman Alqaisi[1], Hani M. El-Zaiat[1,2♦]

[1]Department of Animal and Veterinary Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, P.O. Box 34, 123 Al-Khod, Sultanate of Oman
[2]Department of Animal and Fish Production, Faculty of Agriculture, University of Alexandria, Aflaton St., El-Shatby, P.O.Box 21545, Alexandria, Egypt
♦Corresponding author: hani.elzaiat@alexu.edu.eg

**Abstract**

**Neem (*Azadirachta indica*) belongs to *Meliaceae* family, represented mainly by trees, and widely cultivated and adapted in many tropical regions. The objective of this study was to evaluate the effect of increasing doses of neem seed oil (NSO) on ruminal methane ($CH_4$) formation, diet degradability, and fermentation characteristics after 24 h of *in vitro* incubation. Treatments were randomly designed to four doses of NSO supplemented to the basal diet (0, 20, 40, or 60 ml/kg DM). Increasing NSO dose resulted in a quadratic decrease (P<0.05) in net gas (expressed as ml/g DM and ml/g TDOM) and $CH_4$ (expressed as ml/g TDNDF) production, while $CH_4$ (expressed as ml/g TDOM), acetate and propionate proportions decreased linearly confirming a dose-related effect. A quadratic increase in TDOM and linear increase (P = 0.023) in DNDF, $NH_3$-N concentrations, and total protozoal counts were observed. However, a linear increase (P = 0.009) was found in the ruminal butyrate proportion and partitioning factor as dietary NSO supplementation increased. In conclusion, dietary NSO supplementation mediated some desirable fermentation patterns, reducing ruminal $NH_3$-N concentration and $CH_4$ production with some adverse effects on fiber degradability. However, practical research under long-term conditions is required for further investigation.**

**Key words: neem seed oil, rumen fermentation, feed degradability, methanogenesis**

The worldwide interest in alleviating the methane ($CH_4$) emission from livestock is one of the potential reasons for global warming. Within ruminants, $CH_4$ derived from the enteric fermentation processes is of global concern as one of the major significant greenhouse gases (Hook et al., 2010). The emitted $CH_4$ represents a considerable dissipate of 2–12% of total energy intake for the host animal (Johnson and Johnson, 1995) which could allegedly be redirected towards meat or milk production (Patra and Yu, 2015) and ensure better environmental sustainability as well (Haque, 2018). However, in this regard, the amount of $CH_4$ produced in the rumen is potentially related to the differences in diet ingredients and composition. Thus, dietary intervention could be one of the substantial nutritional strategies to sustainable abatement of $CH_4$ from ruminants (Hristov et al., 2013 a; Gomaa et al., 2018; Salama et al., 2020). However, the continuous attempt to devise proper strategies to alleviate ruminal $CH_4$ formation through dietary modification (Haque, 2018), essential oil, phytogenic feed additives (El-Zaiat and Abdalla, 2019; El-Zaiat et al., 2020; Morsy et al., 2021) and the addition of nitrate or organic acid (El-Zaiat et al., 2014, 2019 a) has recently been practiced. Oilseeds supplementation is considered one of the persistent alleviation strategies and has been incorporated in ruminant feeding to reduce $CH_4$ production and improve the nutritive value of diets therapy to increase animal productivity (Bayat et al., 2018; Lima et al., 2019; Ebeid et al., 2020; El-Nile et al., 2021). The plentiful amounts of triglycerides and unsaturated fatty acids contents in oils such as oleic and linoleic acids (Roy et al., 2017), can be environmentally effective in minimizing ruminal $CH_4$ formation (Machmüller et al., 1998). However, methanogenesis depression is presumably by modifying the organic matter fermentation pathway, fiber degradability, and direct inhibition of methanogens (Johnson and Johnson, 1995). Therefore, several research studies (Hristov et al., 2013 b; Gomaa et al., 2018; Vargas et al., 2020) have been documented that along with the potential effects of oilseeds on ruminal microbes and $CH_4$ formation, there is an increasing interest to emphasize that feed degradability is not hindered (Haque, 2018).

Neem tree (*Azadirachta indica*) belongs to the Meliaceae family widely cultivated in many developing countries with medicinal properties for more than 400 years (Girish and Shankara, 2008). Neem tree parts (such as fruits, leaves, bark, seeds, and roots) were reported to contain phytochemical active components and proven biological, agricultural, and medicinal (antiviral, anti-inflammatory, and antifungal) properties (Lokanadhan et

al., 2012). It was noted that neem seeds are rich in unsaturated fatty acids and bioactive phytochemicals (Djibril et al., 2015), that draw the attention following other oilseeds to exhibit asymmetric attenuation response, in terms of ruminal $CH_4$ formation (El-Zaiat et al., 2021). Nonetheless, there is a paucity of research data investigating the potency of neem seed oil (NSO) on ruminal fermentation including substrate digestion and $CH_4$ production. Therefore, we hypothesize that dietary supplementation with NSO would alter ruminal fermentation functions and feed degradability, and consequently keep $CH_4$ production constant or even decreased. Thus, this study was undertaken to investigate the influence of increasing doses of NSO on fermentation functions, feed degradability, and methanogenesis under *in vitro* conditions.

## Material and methods

This experiment was conducted at the Laboratory of Animal Nutrition, Department of Animal and Fish Production, Faculty of Agriculture, Alexandria University, Egypt. Samples were analyzed at the Department of Animal and Veterinary Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University (Oman), and Animal Production Department, Faculty of Agriculture, Alexandria University (Egypt).

### Neem seed oil fatty acid analysis

The fatty acid concentrations of NSO were determined using gas chromatograph (GC-MS, Shimadzu model QP2010 Ultra, Kyoto, Japan) analysis of the fatty acid methyl esters (FAMEs) derived by methanolic boron trifluoride ($BF_3$) reagent (Besbes et al., 2005; Morrison and Smith, 1964). The Individual FAMEs were identified based on comparing the spectra obtained with appropriate mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition). Individual fatty acid was expressed as g/kg g of FAMEs (Table 2).

### Preparation of rumen inoculum

Ruminal contents were collected from three cows (approximate body weight (BW) of 450 ± 50 kg) freshly slaughtered (Alexandria University slaughterhouse, Alexandria, Egypt) in equal amounts following the procedure described by Mould et al. (2005 a, b). The collected inoculum was pooled and transferred to a pre-warmed thermal flask and immediately transported to the laboratory. Following this, rumen contents were blended in equal proportion for 10 sec, extruded through four layers of cheesecloth to remove feed particles into pre-warmed flasks (39ºC) in a water bath, and maintained under continuous flushing with $CO_2$ until use (Bueno et al., 2005).

### Experimental design and treatments preparation

The experiment was conducted in a completely randomized design (CRD) with four treatments (NSO dose) to identify the role of increasing levels of NSO on ruminal fermentation and $CH_4$ formation. The NSO was purchased from Al Barakah factory, Al Maabilah industrial Area, Seeb, Muscat, Sultanate of Oman. Each fermentation serum bottle was the experimental unit, resulting in six replicates per treatment. The experimental substrate was used as total mixed rations (forage to concentrate ratio at 50:50 on a DM basis) with the forage part consisting of berseem (*Trifolium alexandrinum*) hay. The ingredients and chemical compositions of the substrate and berseem hay used in this experiment are presented in Table 1. The substrate was analyzed for dry matter, organic matter, and crude protein (as 6.25 × N) using the procedures of AOAC (2006). While, the neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van Soest et al. (1991) using an Ankom 200 Fibre Analyzer unit (ANKOM Technology Corporation, Macedon, NY, USA) and expressed exclusive of residual ash.

Table 1. Ingredients and chemical composition of the experimental basal diet (g/kg of dry matter)

| Items | Basal substrate | Berseem hay |
|---|---|---|
| Ingredients | | |
| chopped berseem hay (*Trifolium alexandrinum*) | 500 | – |
| soybean meal | 150 | – |
| ground yellow corn | 345 | – |
| limestone | 1.00 | – |
| sodium chloride | 1.35 | |
| mineral mixtures[1] | 0.85 | |
| vitamin mixtures[2] | 1.80 | |
| Chemical composition | | |
| organic matter | 917 | 885 |
| crude protein | 103 | 135 |
| ether extract | 22 | 27 |
| neutral detergent fibre | 303 | 538 |
| acid detergent fibre | 206 | 359 |
| non-fibre carbohydrates[3] | 488 | 135 |

[1]Each one kg contains per kg: 40 g magnesium, 0.4 g iodide, 15 g copper, 0.15 g cobalt, 50 g zinc, 0.2 g selenium (Multi vita Company, Egypt).
[2]Each one kg contains per kg: 90,000,000 IU vitamin A, 2,500,000 IU vitamin D, 35,000 mg vitamin E (Multi vita Company, Egypt).
[3]Non-fibre carbohydrates calculated as 1000 − (NDF g/kg + CP g/kg + EE g/kg + ash g/kg).

Samples of 500 mg DM of the experimental substrate were weighed and placed into 120 mL glass serum bottles and supplemented with NSO at 0, 20, 40, and 60 ml/kg DM substrate. A total of two incubation runs were performed, each treatment was incubated in six replications, plus four bottles as blanks (rumen fluid and buffer solution only), and the other four bottles as an internal standard (clover hay, rumen fluid, and the buffer solution) in each run according to El-Zaiat and Abdalla (2019). The total serum bottles used were: 64 bottles ((6 replicates × 4 treatments + 4 blanks + 4 internal standard) × 2 incubation runs). Each bottle (pre-heated, 39ºC)

was then injected with 15 ml of fresh rumen fluid and 50 ml of buffer (adjusted pH to 6.80) solution, then flushed with $CO_2$ to ensure anaerobiosis condition. Immediately, all bottles were closed with a rubber stopper followed by sealing with an aluminum cap to prevent gas leakage, gently shaken, and placed in a forced-air incubator at 39ºC (FALC instruments S.R.L., Treviglio, Italy) for 24 h.

Table 2. Fatty acid composition of neem seed oil

| Items | g/kg FAMEs |
|---|---|
| C14:0 | 1.30 |
| C16:0 | 140 |
| C16:1 | 1.70 |
| C18:0 | 164 |
| C18:1 | 563 |
| C18:2 | 75.9 |
| C20:0 | 20.6 |
| C20:1 | 2.30 |
| C22:0 | 4.30 |
| C23:0 | 4.30 |
| Others | 22.6 |
| ΣSFA | 334.5 |
| ΣUSFA | 642.9 |

FAMEs, fatty acid methyl esters; C14:0, Myristic acid; C16:0, Palmitic acid; C16:1, Palmitoleic acid; C18:0, Stearic acid; C18:1, Oleic acid; C18:2, Linoleic acid; C20:0, Arachidic acid, C20:1, 11-Eicosenoic acid; C22:0, Behenic acid; C23:0, Lignoceric acid; ΣSFA, total saturated fatty acid; ΣUSFA, total unsaturated fatty acids.

**Gas production and methane determination**

Incubation lasted 24 h, and net gas production (NGP) of each bottle was measured after 3, 6, 9, 12, and 24 h of incubation by a semi-automatic technique system (Bueno et al., 2005) using a pressure transducer and a data logger (GN200, Sao Paulo, Brazil). The headspace GP was sampled (2 mL) from each bottle at each GP measuring with a 10 mL syringe (med Dawliaico, Assiut, Egypt) and immediately transferred into 10 mL evacuated tubes (BD Vacutainer® Tubes, NJ, USA) which was then analyzed for $CH_4$ concentration by gas chromatography.

The $CH_4$ concentration was determined by GC (Model 2014, Drawell Scientific Instrument Co., Ltd, Shanghai, China) equipped with a Molesieve 5A micro packed column (1 m, 2 mm ID, Ref no. 80440-800; Restek, Bellefonte, PA, USA). The temperatures of the column, injector, and thermal conductivity detector were 50, 150, and 200°C, respectively. Helium was the carrier gas and was added at a constant flow (30 ml/min). The $CH_4$ produced was calculated as follows: $CH_4$ (mL) = (total net GP volume (mL) + bottle headspace (75 mL)) × $CH_4$ concentration (ml/ml). The net GP and $CH_4$ values were calculated by subtracting the blank values produced.

**Determination of rumen fermentation attributes and substrate degradability**

After the termination of incubation time, the fermentation process was terminated by placing the entire bottle in chilled water. After opening the bottles, pH was measured directly using a portable pH meter (model GLP 21; Crison, Barcelona, Spain). Three bottles were assigned to the determination of the truly degraded organic matter and neutral detergent fiber (TDOM and TDNDF, respectively). TDOM and TDNDF were determined by the differences in OM and NDF between the substrate before and the residue after 24 h of fermentation as described by El-Zaiat et al. (2019 b). However, the incubation fluid of the other three bottles was used for determining rumen fermentation end-products (NGP, $CH_4$, volatile fatty acids (VFA), and ammonia nitrogen ($NH_3$-N) production).

A portion of the rumen liquid was collected for $NH_3$-N concentrations determination by spectrophotometer (model Alpha-1101; Labnics Equipment, California, USA) using commercial lab test as described by Konitzer and Voigt (1963). In a glass bottle, a 1 mL subsample of ruminal fluid was mixed with 2 mL of methyl green formalin-saline solution for total protozoa counts following the procedure described by Dehority (1993).

The individual VFA concentrations were measured using a gas chromatograph (Thermo Fisher Scientific, Inc., TRACE1300, Rodano, Milan, Italy) fitted with an AS3800 autosampler and equipped with a capillary column FFAP (0.5 µm film o.d., 0.53 µm i.d., and 30m length; J and W Agilent Technologies Inc., Palo Alto, CA) as described with some modifications described in details by El-Zaiat et al. (2020). Nitrogen at 7 mL/min was used as carrier gas. Air, hydrogen, and nitrogen fluxes (makeup gas) were kept at 450, 40, and 35 mL/min, respectively. A subsample of 1.6 mL of ruminal fluid was mixed with 0.4 mL metaphosphoric acid (25%) and subsequently centrifuged at 4°C at 15,000 × g for 20 min (K1015 Micro Prime; Centurion Scientific Ltd, Stoughton, Chichester, UK). The supernatant (0.1 µL) was injected in a splitless mode for the entire run with 31.35 mL/min of $H_2$ flux (63.432 Pa). Injector and flame ionization detector (FID) temperatures were held isothermally at 250°C. The oven heating slope was 80°C (1 min), 120°C (20°C/min for 3 min), and 205°C (10°C/min for 2 min). The partitioning factor (PF) as an indicator of microbial protein was determined as described by Blümmel et al. (1997) as the ratio of IVTDOM (mg) and net gas volume (ml).

**Statistical analyses**

All data were analyzed as a CRD using the PROC MIXED procedure of SAS (version 9.1, SAS Inst. Inc., 2002, Cary, NC) and subjected to one-way ANOVA and regression to test the effects of incremental levels of NSO on the measured variables. The effect of supplementing NSO levels to the diet was tested by orthogonal polynomials contrast (linear and quadratic responses) for the effects of incremental levels of NSO supplementation on the diet. Among the treatment means, multiple compari-

sons were performed using a Tukey test to compare the differences, and the results were considered statistically significant when P<0.05.

## Results

### Neem seed oil fatty acid composition

In the present study, the fatty acids in NSO range from C14:0 to C23:0 (Table 2). NSO is a good source of total unsaturated fatty acids accounting for approximately 64% (USFA) in the form of 0.17% palmitoleic acid (C16:1), 56% oleic acid (C18:1), 7.59% linoleic acid (C18:2) and 0.23% eicosenoic acid (C20:1) and around 33% is saturated fatty acids (SFA) and 2.3% other fatty acids.

### Total net gas and methane production and feed degradability

The net GP (expressed as ml/g DM and ml/kg TDOM) and $CH_4$ (expressed as ml/kg TDNDF) decreased quadratically (P<0.05), while $CH_4$ (expressed as ml/g DM and ml/g TDOM) decreased linearly (P<0.05) as the dose of NSO increased in the diet (Table 3). In addition, a quadratic increase in TDOM (expressed as g/kg) upon increasing NSO dose was observed. As NSO doses increased in the diet, a linear increase (P = 0.023) in TDNDF and increases in partitioning factor (mg TDOM/ml gas production for 24 h) were also observed (Table 4).

### *In vitro* ruminal fermentation functions

Ruminal acetate (C2) and propionate (C3) proportions decreased linearly (P<0.05) upon the increasing levels of NSO in the diet (Table 5). However, a linear increase (P = 0.009) was found in the ruminal butyrate proportion as dietary NSO supplementation increased. However, the ruminal pH, isobutyrate (iso-C4), valerate (C5), isovalerate (iso-C5), acetate: propionate ratio (C2:C3), and total VFA concentration were similar among treatments (P>0.05). As the level of NSO in the diets increased, $NH_3$-N concentrations and total protozoal counts linearly decreased (P<0.05, Table 5).

Table 3. Effects of graded doses of neem seed oil supplementation to the basal substrate on *in vitro* ruminal gas and methane production

| Neem seed oil levels (ml/kg DM substrate) | Net GP (ml/g DM) | Net $CH_4$ (ml/g DM) | Net GP (ml/kg TDOM) | $CH_4$ (ml/g) | |
|---|---|---|---|---|---|
| | | | | TDOM | TDNDF |
| 0 | 138 | 11.11 | 223 | 17.9 | 36.1 |
| 20 | 122 | 9.54 | 205 | 16.1 | 29.8 |
| 40 | 112 | 7.44 | 178 | 11.9 | 21.3 |
| 60 | 115 | 7.40 | 179 | 11.6 | 21.6 |
| SEM | 0.99 | 0.25 | 2.04 | 0.47 | 0.64 |
| Orthogonal polynomials (P-value) | | | | | |
| linear | <0.001 | 0.005 | <0.001 | 0.028 | <0.001 |
| quadratic | <0.001 | 0.140 | 0.029 | 0.425 | 0.017 |

GP, gas production; $CH_4$, methane; TDOM, truly degraded organic matter; TDNDF, truly degraded neutral detergent fibre.
SEM, standard error of the mean.

Table 4. Effects of graded doses of neem seed oil supplementation to the basal substrate on *in vitro* ruminal feed degradability and partitioning factor

| Neem seed oil levels (ml/kg DM substrate) | Degradability (g/kg) | | Partitioning factor (mg TDOM/ml gas production) |
|---|---|---|---|
| | TDOM | TDNDF | |
| 0 | 619 | 308 | 0.75 |
| 20 | 595 | 321 | 0.87 |
| 40 | 626 | 345 | 1.06 |
| 60 | 641 | 342 | 1.00 |
| SEM | 3.74 | 4.56 | 0.05 |
| Orthogonal polynomials (P-value) | | | |
| linear | 0.105 | 0.023 | 0.017 |
| quadratic | 0.017 | 0.371 | 0.125 |

TDOM, truly degraded organic matter; TDNDF, truly degraded neutral detergent fiber; SEM, standard error of the mean.

Table 5. Effects of graded doses of neem seed oil supplementation to the basal substrate on *in vitro* ruminal volatile fatty acids (VFA), pH, ammonia nitrogen ($NH_3$-N) concentration, and protozoa abundance

| Neem seed oil levels (ml/kg DM substrate) | VFA (mol/100mol) | | | | | | | Total (Mm) | pH | $NH_3$-N, (mg/dL) | Total protozoa, ($10^5$ cell/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *C2* | *C3* | *C4* | *iC4* | *C5* | *iC5* | *C2:C3* ratio | | | | |
| 0 | 71.3 | 17.64 | 7.66 | 1.77 | 2.01 | 0.91 | 4.04 | 101.3 | 6.83 | 18.90 | 4.49 |
| 20 | 64.97 | 15.68 | 11.17 | 1.59 | 1.99 | 0.91 | 4.15 | 96.31 | 6.91 | 15.60 | 4.16 |
| 40 | 61.84 | 14.78 | 11.54 | 1.32 | 1.78 | 0.82 | 4.16 | 92.16 | 7.00 | 15.20 | 3.96 |
| 60 | 55.84 | 13.72 | 13.17 | 1.37 | 1.58 | 0.72 | 4.08 | 86.39 | 6.97 | 15.10 | 3.29 |
| SEM | 0.37 | 0.15 | 0.25 | 0.05 | 0.03 | 0.01 | 0.04 | 0.44 | 0.05 | 0.13 | 0.09 |
| Orthogonal polynomials (P-value) | | | | | | | | | | | |
| linear | 0.008 | 0.029 | 0.009 | 0.995 | 0.636 | 0.515 | 0.640 | 0.105 | 0.701 | <0.001 | 0.005 |
| quadratic | 0.104 | 0.228 | 0.508 | 0.441 | 0.428 | 0.389 | 0.871 | 0.601 | 0.606 | 0.301 | 0.401 |

VFA, volatile fatty acids; *C2*, acetate; *C3*, propionate; *C4*, butyrate; *iC4*, isobutyrate; *C5*, valerate; *iC5*, isovalerate; *C2:C3*, acetate: propionate ratio; $NH_3$-N, ruminal ammonia nitrogen; SEM, standard error of the mean.

## Discussion

In the present study, diets supplemented with graded doses of NSO resulted in an apparent variation in total net gas and $CH_4$ production. Differences observed with ruminal fermentation and feed degradability may possibly be explained by differences in dose, dietary composition, and controlled *in vitro* conditions used. In addition, the potential effect of vegetable oils supplemented to the diet on abatement of $CH_4$ coincided with previous *in vivo* (Mao et al., 2010; Bayat et al., 2018; Lima et al., 2019) and *in vitro* (Dey et al., 2021) research findings. Diet supplemented with NSO affected net GP, feed degradability, and ruminal fermentation in a dose-dependent manner. Linear decrease in $CH_4$ production caused by NSO was achieved with a quadratic response in TDOM, which may be explained by the potency of their fatty acids content likely to create notable changes in the microbial community that reflected in the ruminal fermentation profile (El-Zaiat et al., 2021). Whilst, these responses are mostly associated with NSO higher amount of USFA and their interaction between available feed or substrate ingredients and rumen microbial activities throughout the fermentation process (Wu et al., 2016).

The net GP remained similar in lower doses (20 and 40 ml/kg DM substrate); however, it declined with higher doses (40 and 60 ml/kg DM substrate). The decrease in total net GP and $CH_4$ could be due to inhibition of several microbial populations in the rumen with oils supplementation (Mbiriri et al., 2015). According to Piluzza et al. (2014), the reduction in fiber degradation is associated with a decline in available $H_2$ for ruminal methanogens, thereby decreasing $CH_4$ formation. The higher TDOM and TDNDF and consequently net GP and methanogenesis promoted by NSO might be due to the direct inhibitory effects on ruminal microorganisms (Maia et al., 2006) and to indirect potency by covering the particles of feed (Kozloski, 2009). Therefore, supplementation with NSO may negatively affect rumen microbiota and alter fermentation end-products by preventing of feed microbial colonization and subsequently restricted the microbial enzymes penetration to feed particles.

Despite the antimicrobial activity of NSO (Lokanadhan et al., 2012), an incremental dose of NSO did not exhibit any reduction in total VFA indicating no adverse effects of tested doses on fermentation parameters. The absence of effect of NSO on total VFA concentration could be favorable since it was associated with reduced $NH_3$-N concentrations, protozoa number and $CH_4$ production, and changes in the proportions of individual VFA (Ebeid et al., 2020). Additionally, the lack of effects of NSO on total VFA concentration in the rumen is concomitant with the unchanged pH, revealing normal ruminal conditions (Yang et al., 2009). The potential of NSO to reduce $CH_4$ and $NH_3$-N production was concomitant with the variations in degradability and individual VFA production (Demirtas et al., 2019).

The decrease in C2 and C3 proportion in higher doses might be attributed to the toxic antimicrobial effect of oils, which was also explained by the linear increase in TDNDF, which might be attributed to the negative potency of enzymes responsible for fiber degradation in the rumen (Patra, 2013). In addition, the adverse effect on NDF degradability may partially be attributed to the higher amount of polyunsaturated fatty acids (USFA) in NSO (Wu et al., 2016). The observed deterioration of fiber degradability might be due to the reduction in protozoa abundance (El-Zaiat and Abdalla, 2019) therein concurring with lower C2 proportions. The critical sensitivity of C4-producing bacteria to poly USFA in NSO (Vargas et al., 2020) could explain the linear increase in C4 output with incremental doses of NSO, which was in agreement with other studies (Eugène et al., 2008; Vargas et al., 2020). However, alteration in fermentation functions by reducing ruminal $NH_3$-N accumulation indicates more microbial growth as evidenced by higher partitioning factor (PF) values in our study. Additionally, a decrease in ruminal $NH_3$-N concentrations might be attributed to lower protozoa abundance due to the antiprotozoal properties of neem (Patra et al., 2006).

The reduction in ruminal $NH_3$-N concentration output with dietary oils supplementation could be environmentally advantageous attenuating N emissions from farm animals, thereby alleviating environmental pollution. In our study, the reduction in total protozoa abundance and methanogenesis in the rumen which is mostly associated with oils inhibitory effects considering the changes in the ruminal microbiota was in line with other studies (Patra et al., 2017; Broucek et al., 2018; Nur Atikah et al., 2018). In NSO, oleic acid was found to be the most abundant USFA, which could be responsible for the depression of $CH_4$ production that may be attributed to the toxicity potential of USFA on ruminal protozoa and their associated methanogens (Vargas et al., 2020).

Higher PF indicates that NSO supplementation partitioned more energy into microbial proliferation and less into methanogenesis. Phytogenic feed additives are capable to reduce $CH_4$ production in the rumen due to their negative potential on proliferation of methanogens, through the significant changes in the nutrient composition and balance of the diet (García-González et al., 2008). Ruminal biohydrogenation of USFAs has been identified to compete with the $CH_4$ formation pathway for a considerable amount of metabolic $H_2$ (Toprak et al., 2015). Thus, higher USFAs in NSO such as oleic acid, stearic acid, palmitic acid, linoleic acid, and arachidic acid (Khanam et al., 2017) could be responsible for the direct depression of methanogens via biohydrogenation process as one of $H_2$ sink strategy, thereby diverted $H_2$ from methanogenesis (Lima et al., 2019). We have not determined the main active compounds while the inhibitory effects of NSO on the activity of methanogens might be not only associated with feed degradability also due to the presence of active components or secondary metabolites in NO seeds.

## Conclusions

Dietary supplementation with incremental doses of NSO mediated some desirable ruminal fermentation kinetics. Feed degradability improved while protozoa number, $NH_3$-N concentration, and $CH_4$ production were decreased by supplementing NSO to diet. These findings reflect the great ability of NSO for shifting nitrogen excretion from urine to feces, which could have a beneficial implication from both nutritional and environmental views. However, further practical research is recommended and should focus on different roughage levels and the responses in nutrients intake and digestibility, as well as animal health under *in vivo* long-term effect, have to be evaluated.

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