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The effect of dietary nitrate and canola oil alone or in combination on fermentation, digesta kinetics and methane emissions from cattle

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ABSTRACT

Nitrate and lipids have been recognized as effective dietary additives to reduce enteric methane (CH_4) production. The objective of this experiment was to evaluate the effects of nitrate (NO_3^-) and canola oil, alone or in combination, on enteric CH_4, volatile fatty acid (VFA) concentrations, digesta kinetics and outflow of DM and microbial non-ammonia nitrogen (MicNAN) from the rumen of cattle. Four rumen-cannulated steers were used in the experiment which was designed as 4 × 4 Latin Square with four 21-d periods and four treatments. Dietary treatments consisted of a control diet (CON: 400 g/kg lucerne chaff and 600 g/kg barley grain), NO_3^- (CON + 20 g NO_3^-/kg), O (CON + 50 g canola oil/kg), and NO_3^- + O (CON + 20 g NO_3^-/kg + 50 g canola oil/kg) with all inclusions expressed as g/kg as-fed. Exogenous markers (Co-EDTA, Yb-acetate and $^{15}NH_4Cl$) were continuously infused into the rumen over 4 d to estimate digesta flow and rumen N outflow while whole tract digestibility (DMD) was determined using chromic oxide. Compared with the CON diet, feeding the NO_3^- + O diet reduced ($P < 0.01$) methane yield (MY, g CH_4/kg DMI) by 25%, daily methane production (DMP, g CH_4/d) by 26% ($P < 0.01$) and the rumen mean retention time (MRT; $P < 0.05$). Nitrate containing diets reduced DMD ($P < 0.01$). Total VFA did not differ between treatments ($P > 0.05$) but NO_3^--containing diets increased acetate proportion ($P < 0.01$) whereas feeding the O diet increased propionate proportion ($P < 0.01$). Oil-containing diets reduced rumen volume ($P < 0.01$). The rumen protozoa concentration was reduced by including NO_3^- and canola oil alone or in combination in the diet of cattle ($P < 0.05$). This experiment demonstrates that feeding NO_3^- + O has a synergistic effect on reducing methanogenesis from beef cattle which is consistent with NO_3^- and canola oil having complementary mechanisms for suppressing enteric CH_4 production. Reducing methanogenesis by feeding NO_3^- + O in this experiment did not improve the flow of MicNAN from the rumen (g MicNAN/d), microbial growth efficiency (g MicNAN/digestible organic matter intake, DOMI) or the proportion of microbial N derived from rumen NH_3.

Abbreviations: CH_4, methane; DMI, dry matter intake; DMP, daily methane production; DMD, dry matter digestibility; DMADR, dry matter apparently digested in the rumen; DOMI, digestible organic matter intake; FP, fluid phase; GHG, greenhouse gases; LW, liveweight; MicNAN, microbial non-ammonia nitrogen; MRT, mean retention time; MY, methane yield NAN, non-ammonia nitrogen; NH_3, ammonia; NO_3^-, nitrate; NO_2^-, nitrite; OMDR, organic matter as digested in the rumen; PP, particulate phase; TD, true digesta; VFA, volatile fatty acid

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1. Introduction

Methane accounts for 43% of the greenhouse gas (GHG) emissions from livestock with the remaining emission released almost equally as nitrous oxide (29%) or carbon dioxide (CO_2; 27%) (Herrero et al., 2016). Hydrogen (H_2) produced in the rumen during microbial fermentation of carbohydrates provides electrons that reduce CO_2 to CH_4. One strategy to reduce GHG emissions in ruminants is the manipulation of rumen fermentation by using dietary additives. Dietary nitrate (NO_3^-) reduces methanogenesis by acting as a H_2 sink in the rumen (Lee and Beauchemin, 2014). Nitrate is an inorganic anion with strong potential to act as an oxidising agent. When present in the rumen, NO_3^- has a higher affinity for H_2 than does CO_2, and therefore CH_4 production is decreased (Jones, 1972). Nitrate is firstly reduced to nitrite (NO_2^-) and then to ammonia (NH_3), which is energetically more favourable than the reduction of CO_2 to CH_4 and reduces H_2 availability for methanogens (Ungerfeld and Kohn, 2006; Latham et al., 2016).

The production of methaemoglobinaemia that results from NO_2^- generated in the rumen and absorbed into the bloodstream is one of the major practical limitations to adoption of NO_3^- supplementation for ruminant livestock (Bruning-Fann and Kaneene, 1993; Nolan et al., 2016). However, several strategies to reduce the risk of NO_2^- poisoning are being developed (de Raphélis-Soissan et al., 2017a,b; Lee et al., 2017). In addition to reducing the loss of energy in CH_4, NO_3^- can replace urea as a non-protein N source for rumen microorganisms (Leng, 2008; Goopy and Hegarty, 2018) and its inclusion in lick blocks to get carbon credits has been approved in Australia (DoE, 2013).

Inclusion of lipids in the diet of ruminants is another option to reduce enteric CH_4 emissions by supressing the growth of methanogens (Patra and Yu, 2012), or by lowering ruminal fermentability of the diet and to a lesser degree through hydrogenation of unsaturated fats (Beauchemin and McGinn, 2006). The inhibitory effect of lipid supplementation on CH_4 emissions is not transitory but persists over time (Moate et al., 2011; Guyader et al., 2016). More recent studies have shown that the combination of dietary NO_3^- and dietary oils in cattle has an additive and longer-term CH_4 inhibiting effect than either treatment on its own (Guyader et al., 2015; Duthie et al., 2018).

A faster passage rate of rumen digesta is associated with an increase in both microbial growth and the efficiency of microbial synthesis *in vitro* (Meng et al., 1999) and *in vivo* (Li et al., 2000). In addition, a shorter rumen mean retention time (MRT) and a smaller rumen volume are associated with a reduction of CH_4 emission rate in sheep (Pinares-Patiño et al., 2011a; Goopy et al., 2014). Dietary NO_3^- has been shown to reduce total MRT and to increase microbial N outflow in sheep while reducing CH_4 emissions (Nguyen et al., 2016). Similarly, the inclusion of oils rich in polyunsaturated fatty acids in the diet of ruminants has been shown to increase microbial synthesis and non-ammonia N flow particularly when ruminal protozoal number in the rumen are reduced (Doreau and Ferlay, 1995; Ueda et al., 2003), and consequently a reduction in enteric CH_4 production is also expected (Newbold et al., 2015).

While results on the effect of NO_3^- and lipids in combination on CH_4 production and performance of cattle have been recently published (Guyader et al., 2015, 2016; Popova et al., 2018), there is little information on the effect of NO_3^- and canola oil in combination on digesta kinetics or microbial non-ammonia nitrogen (MicNAN) outflow in cattle. This experiment evaluated the effects of NO_3^- and canola oil, alone or in combination, on CH_4 production and VFA balance, digesta kinetics, outflow of microbial N from the rumen of cattle and microbial growth efficiency (g MicNAN/DOMI). We hypothesised that feeding NO_3^- in combination with canola oil ($NO_3^- + O$) may not only lead to a change in energy availability, but also to differences in microbial N supply that could affect animal performance.

2. Materials and methods

All procedures involving animals were performed in accordance with the University of New England Animal Ethics Committee (AEC approval no. 15/104).

2.1. Animals, diets and experimental design

Four mature rumen-cannulated steers were used in the experiment, which was designed as a 4 × 4 Latin square with four 21-d periods and four dietary treatments in a 2 × 2 factorial arrangement. Steers (698 ± 21 kg, mean ± SD) were randomly allocated to the dietary treatment and progressively adapted to the diet. Each steer was offered one of the four dietary treatments in each period. All diets were fed at 1% group average liveweight (LW) being 7.5 kg fresh feed/d to each animal. The control diet was a blended chaff mixture (40% lucerne chaff; 60% rolled barley grain) fed alone (Control; CON) or with inclusion of 20 g NO_3^-/kg (NO_3^-), provided as 31.4 g/kg calcium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway). The third treatment (O) had 50 g canola oil /kg added to the chaff and the final treatment ($NO_3^- + O$) contained 20 g NO_3^-/kg and 50 g canola oil/kg in combination, with all inclusions expressed as g/kg as fed. Each experimental period was preceded by 4 d of introduction to the experimental diet where 50% of the CON diet and 50% of the experimental diet was offered on Day 1 and Day 2; 25% of CON diet and 75% of the experimental diet was offered on Day 3 and Day 4, and from Day 5 to Day 21 cattle were fed the experimental diets. While diets were not isonitrogenous, the level of crude protein (CP) relative to metabolisable energy (ME) in the control diet (1.9 g N/MJ ME) was within that required to maximise rumen microbial activity (1.9 g N/MJ ME, Freer et al., 2007) so the additional N provided by NO_3^- was unlikely to stimulate microbial growth or fermentation. Diets were prepared in a ribbon mixer every two weeks by spraying dissolved Bolifor or canola oil onto the chaffed lucerne hay and barley grain so that these additives could not settle out when fed in each period. Animals were offered 7.5 kg/d of their experimental diet in two meals each day (at 0930 and 1700 h). During days when markers were infused the daily ration was offered in three equal portions at 0730, 1500 and 2300 h. Over the 3 day of infusions, reticular and

Table 1
Analysed nutrient composition of the experimental diets fed to cattle (g/kg DM).

	CON[1]	NO_3^-	O	NO_3^- + O
DM	917	901	923	905
DM digestibility	778	773	775	768
Digestible organic matter	770	763	768	758
Organic Matter	950	940	950	940
Neutral detergent fibre	303	290	298	290
Acid detergent fibre	155	150	158	150
Crude protein	140	167	138	166
Crude fat	19	19	73	80
Metabolisable energy (MJ/kg DM)	12.0	11.9	13.4	13.5
Gross energy (MJ/kg DM)	18.6	18.1	19.6	19.2

[1]CON = control; NO_3^- = CON + 20 g NO_3^-/kg as-fed basis; O = CON + 50 g canola oil/kg as-fed basis; NO_3^- + O CON plus 20 g NO_3^-/kg as-fed basis and 50 g canola oil/kg as-fed basis.

rumen samples were scheduled to provide samples matching each hour over the 8 h interval between feeds, associated with feeding 3 times per day. This was done to minimize diurnal fluctuation in fermentation and provide a very stable rumen fermentation and marker concentration in digesta samples.

Individual feed offerings and refusals were weighed and recorded daily throughout the experiment and mean daily intake was calculated. One sample of each diet was collected during each experimental period and stored at −20 °C. A final composite sample consisting of 4 samples/diet was subsampled and analysed for chemical composition (Table 1) at the NSW DPI Feed Quality Service, Wagga Wagga Agriculture Institute. The methods described by AFIA (2014) used specifically were acid detergent fibre, neutral detergent fibre, DMD of the feed and crude fat estimated by petroleum ether extraction. Metabolisable energy was calculated based on method 2.2R. Feed CP was determined by Dumas combustion (AOAC, 990.03). Energy values were determined using a bomb calorimeter (Method ID LMOP 2–1118). Diets were formulated to meet the maintenance requirements of beef cattle (Freer et al., 2007). From Day 1 to Day 13 of each period animals were housed individually in pens (3 m x 2 m) equipped with a feeder and water bowl. Before the morning feeding on Day 14, animals were moved to four open circuit respiration chambers for measurement of CH_4 emissions over 24 h. On Day 15, animals were weighed, background rumen digesta samples were collected, cannula bungs were equipped with infusion lines and animals were constrained individually in smaller pens (2 m × 1.5 m) for the following 5 d. Digesta marker infusion and rumen-reticulum sampling were performed from Day 16 to Day 20. Before the morning feeding on Day 21, animals were again moved to respiration chambers for a second 24 h period of CH_4 emission measurement and the final LW of animals was recorded.

2.2. Infusion of flow markers (Co-EDTA, Yb-acetate and $^{15}NH_4Cl$)

Animals received a continuous intraruminal infusion of ytterbium acetate (14.64 g/d of Yb, Inframat, 99.99%), ^{15}N-ammonium chloride (3.82 g/d of ^{15}N, 99.9 atoms %, ICON Isotopes, MI, USA) and Co-EDTA (16.70 g/d of Co, AVA Chemicals, Mumbai, India) for 4 d, commencing at 0900 h on Day 16 and ceasing at 0900 h on Day 20. Markers were dissolved in MilliQ® water (5.7 mg Co/L, 1.1 mg ^{15}N/L and 4.9 mg Yb/L) and continuously infused at rates of 0.19 ml/min (Co), 0.21 ml/min (Yb) and 0.20 ml/min (^{15}N), using a peristaltic pump (Masterflex L/S 7528-30, Cole-Parmer, USA). Before beginning the infusions in each period, two samples of mixed rumen contents were taken from each steer and stored at −20 °C for later determination of background ^{15}N, Co and Yb concentrations. Chromic oxide was well mixed in the total diet and fed daily from Day 10 to Day 21 in each experimental period to assess whole tract digestibility.

2.3. Sampling for digesta flow, rumen bacteria, VFA concentrations, rumen ammonia and protozoal enumeration

Reticular samples (7 per animal) were collected *via* the rumen cannula at 1000 and 2200 h on Day 18, at 0730, 1600 and 1900 h on Day 19, 0400, and 0900 h on Day 20. This was at least 14 d after animals had been adapted to their full diet which is considered sufficient to stabilize the rumen biota (Henderson et al., 2015). Reticular sampling was conducted as described by Hristov (2007). Samples were collected using a 30 cm × 65 mm PVC pipe capped at both ends with a 70 mm × 35 mm port cut into the side. The sampling pipe was fitted with a wider diameter sleeve that could be slipped up or down the collection tube to cover or expose the sampling port. The sampler was closed, held in the right hand and introduced through the rumen cannula and pushed forward into the reticulum until the proximal wall of the reticulum could be felt. The sleeve was then pulled up to expose the sample port and the sampler moved around within the reticulum, to allow digesta to enter the sampler. Then the sleeve was lowered to cover the port before the sampler was removed from the rumen. The reticular digesta was then tipped into a beaker and mixed thoroughly before a 40 ml subsample was collected and stored at −20 °C.

Rumen digesta samples (10 per animal) were collected *via* the rumen cannula using the same technique as for reticular digesta sampling. Rumen background samples were collected at 1000 and 1300 h on Day 15. Samples were taken at 1000 and 2200 h on Day 18, at 0730, 1600 and 1900 h on Day 19 and at 0400, 0900 1300, 1500 and 1900 h on Day 20. The last 3 samples taken on Day 20 were to enable the post-infusion decline in marker concentrations to be monitored. At every sampling time, a 100 ml of rumen digesta

was collected. A 50 ml subsample of whole digesta was stored at −20 °C. A second 50 ml subsample was filtered through a double layer of cheesecloth muslin into a beaker. Rumen pH was measured immediately using a portable pH meter (Ecoscan pH6, Eutech Instruments Singapore). A further 10 ml subsample of rumen fluid was taken and acidified with 0.3 ml of 18 M sulfuric acid and then frozen at −20 °C for subsequent VFA and NH_3 analyses.

Isolation of bacteria was performed as described in Hegarty et al. (1994). A final 15 ml rumen fluid subsample was centrifuged at 18000*g* for 20 min in a refrigerated centrifuge (Velocity 30R, Dynamica Pty Ltd, Australia) and the supernatant liquid was discarded. The bacterial pellet was rinsed with 25 ml of MilliQ® water and transferred to another centrifuge tube. These bacteria were re-suspended in MilliQ® water and again centrifuged. This washing procedure was repeated 2 more times. The final pure bacteria pellet was taken up in 3–5 ml of MilliQ® water, frozen at −20 °C and then freeze-dried for N and isotope analysis. Freeze-dried samples of rumen bacteria (1 ± 0.1 mg) with an estimated N content of 80 μg according to previous calculations based on N content in bacterial DM, were weighed into 8 × 5 mm tin capsules prior to analysis (Sercon Limited, Gateway, Crewe, Cheshire, UK). Rumen $^{15}NH_3$-N from rumen fluid was recovered by diffusion from thawed rumen fluid samples in clean sealable glass bottles (McCartney bottle, Wide Mouth, 28 ml) as described by de Raphélis-Soissan et al. (2017).

The abundance of ^{15}N in rumen bacteria and rumen NH_3-N was analyzed with a Sercon 20–22 continuous flow isotope-ratio mass spectrometer connected to an ANCA-GSL sample preparation unit (Sercon Limited, Gateway, Crewe, Cheshire, UK). Irreversible loss rate (ILR) of rumen NH_3 was calculated by dividing the average (plateau) enrichment of the 10 rumen NH_3 samples from Day 18 to Day 21 by the infusion rate of ^{15}N-NH_3 (mmoles ^{15}N/min) as described by Nolan and Leng (1974).

Frozen samples of acidified rumen fluid were thawed at room temperature and centrifuged at 15000 *g* for 10 min. A 0.5 ml subsample of supernatant was analysed for ruminal VFA concentrations by gas chromatography using isocaproic acid as the internal standard with a Varian CP-3800 chromatographer and Varian Star integration software as described by Nolan et al. (2010). A second 0.5 ml subsample of supernatant was diluted (1 in 20) in MilliQ® water and rumen NH_3-N was determined by a modified Berthelot reaction using a continuous flow analyser (SAN + +, Skalar, Breda, Netherlands).

At the time of collection, a 4 ml subsample of rumen fluid was placed in wide-neck McCartney bottle containing 16 ml of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoa were counted with a Fuchs-Rosenthal optical counting chamber (0.0625 mm^2 and 0.2 mm depth) using a staining technique based on the procedure of Dehority (1984). Protozoa were differentiated into Isotrichidae and Entodiniomorph families and by size into large (> 100 μm) and small (< 100 μm) protozoa.

2.4. Dry matter content, digestibility and digesta kinetics

Dry matter (DM) content of feed, rumen digesta, reticulum digesta and faecal samples were determined by drying at 65 °C in a fan-forced oven to a constant weight and then ground through a 1 mm sieve.

Total tract DMD was estimated using DM intake (DMI) results and faecal concentration of the indigestible marker Cr_2O_3. Fifteen grams of chromic oxide (10.2 g Cr) was mixed onto the feed of individual animals. The marker was provided twice daily (7.5 g Cr_2O_3 x time at 07.30 and 17.00) from d 10 to d 14 during the adaptation period and then 3 times daily (5 g Cr_2O_3 × time at 0730, 1500 and 2300 h), from Day 15 to Day 21 during the infusion period. Although most diets were fully consumed, if any feed was refused, the daily Cr intake was assumed to drop by the same proportion. Faecal samples (approximately 55 g of wet weight) were collected twice daily (0900 and 1730 h) from the clean floor of each pen from Day 10 to Day 14 and three times a day (0730, 1500 and 2300 h) from Day 15 to Day 20. An additional faecal sample was collected prior to commencing Cr_2O_3 feeding in each period and was used to determine the background Cr concentration. Chromic oxide was assumed to be completely indigestible, and the digestibility of DM was calculated as follows:

DMD = 1- [Cr intake (mg/d)/DMI (kg/d)]/Cr in faeces (mg/kg of DM)

Small amounts (< 10 g) of the ground feed, faecal and digesta samples containing the markers were analyzed for Cr concentration using a Bruker Tracer III-V portable X-ray Fluorescence (pXRF) with associated software (Bruker Corp., Billerica, Massachusetts, USA). The procedure for measuring markers concentrations using XRF calibration curves has been published by Barnett et al. (2016).

The kinetics of rumen digesta were determined from the concentrations of markers (Co and Yb) in the ten samples of rumen contents collected during the infusion period (Day 18 – Day 20 as previously chronicled) and in the three samples from after the infusions ceased (Day 21). Concentration versus time data were fitted using non-linear curve fitting algorithms of WinSAAM models (Stefanovski et al., 2003) with one compartment representing either rumen fluid or rumen particulate DM. With knowledge of the rates of infusion of Co (fluid marker) the parameters for the curves of best fit gave estimates of the rumen fluid volume (litres) and the outflow rates of fluid (litres/d).

2.5. Preparation of reticulum digesta samples and digesta flow calculations

A single bulked sample of reticular digesta was made for each animal in each period by mixing an equal weight of material from each of the 7 samples. Sodium tetraborate (1 g/16 ml sample) was added to increase sample pH and facilitate removal of NH_3. The mass of sodium tetraborate added was recorded and subtracted when determining net DM content. The bulked sample was fractionated into a mostly fluid phase (FP) and a mostly particulate phase (PP) by allowing the digesta to settle. A subsample of each phase was taken, freeze dried and ground through a 1 mm sieve for marker concentration analysis by inductively coupled plasma

optical emission spectroscopy (ICP-OES; Varian Vista Radial MPX, Varian Medical Systems, Palo Alto, California, USA). Reticular digesta flow was calculated using the double marker method of Faichney (1992). Due to their high concentrations, reticular markers in Animal 4 in Period 3 for O diet and Animal 1 in Period 3 for NO_3^- + O diet were determined using a Bruker Tracer III-V portable XRF. All other calculations were done using marker concentrations estimated by ICP-OES. Marker concentration in both FP and PP, corrected for marker concentration in background samples, were used for calculation of reconstitution factors, *i.e.* the number of units of FP that must be added (or removed from) one unit of PP to obtain true digesta (TD). Dry matter apparently digested in the rumen (DMADR) was calculated by deducting outflow of DM from DMI. The ^{15}N, enrichment ratio, enrichment in TD/enrichment in bacterial was used to determine the fraction of non-ammonia N (NAN) flow in total NAN leaving the rumen and then microbial NAN outflow (MicNAN). Microbial NAN outflow was calculated by multiplying TD flow and NAN content in TD (mg N/kg TD) by the enrichment ratio. Organic matter (OM) digested in the rumen (OMDR) was estimated assuming 950 g/kg OM in DMADR. Microbial NAN (MicNAN) being microbial NAN flow and digestible OM intake (DOMI) were used to estimate microbial growth efficiency as g MicNAN/kg DOMI (Broderick and Merchen, 1992).

2.6. Measurements of methane production

In each period, daily CH_4 production (DMP) of the cattle was estimated over 24 h when they were confined in open circuit respiration chambers with data averaged for the 2 measures for each animal in each period. Two 24 h measures separated by time were chosen as the correlation between consecutive days is very high but declines over longer period (Pinares-Patiño et al., 2013; Herd et al., 2014; Donoghue et al., 2016). Consequently making 2 measurements several weeks apart assists in getting a more correct estimate of the long term emission rate of an animal. During Period 1, DMP was only measured on Day 13. For Periods 2, 3 and 4 DMP was measured on Day 13 and Day 21. Chambers were opened and resealed daily at 0900 and 1700 h when cattle were fed. Recovery of CH_4 through the chambers was checked pre-measurement and post-measurement by introducing pure CH_4 at a known rate *via* a mass flow controller (Smart Trak 2 Series 100, Sierra Instruments, Monterey, CA, USA) and a Servomex analyser was used to quantify CH_4 concentration data. All daily CH_4 emission data was corrected for 100% CH_4 recovery (mean 108% ± 1.8%). We cannot readily explain why recovery exceeded 100%, but this would not affect the detection of any treatment effects but would have influenced estimates of DMP (g/d) and so CH_4 yield (g/kg DMI). Full details regarding the use of these open-circuit respiration chambers at Armidale (UNE) and CH_4 measurements protocols have been described by Hegarty et al. (2012).

2.7. Statistical analysis

Variables with several data points were averaged per animal and per Period and the effect of dietary treatments was determined using a linear mixed-model that included the random effect of animal (n = 4) and the fixed effects of Period (n = 4), Nitrate (CON and O versus NO_3^- and NO_3^- + O), Oil (CON and NO_3^- versus O and NO_3^- + O), and the interaction Nitrate × Oil. All statistical tests were carried out in R 3.3.1 (R Core Team, 2016) using the packages *nlme* and *lme4* for linear mixed effects models. Data on rumen protozoa concentration was log transformed before analysis. During the course of the experiment, animal 4 (NO_3^- + O diet in Period 1) left a large feed refusal while it was inside of the respiration chamber and animal 1 (NO_3^- diet in Period 2) lost the cannula bung before it was scheduled to go into the chamber and lost a large amount of rumen fluid. Thus, observations of those animals for DMP and CH_4 yield (MY) were removed from the CH_4 dataset for those periods. Because of marker infusion problems observations from animal 3 in Period 1 for O diet and animal 4 in Period 1 for NO_3^- + O diet were removed from the analyses of digesta flow. Homogeneity of variance and normal distribution were tested using residuals plots and Shapiro-Wilk test. Main effects of Nitrate, Oil and the interaction Nitrate × Oil were tested using contrasts and multiple comparisons (lsmeans procedure) were performed when the interaction Nitrate × Oil was tested significant. Differences with $P < 0.05$ were regarded as significant and $P < 0.10$ as a trend.

3. Results

3.1. Feed intake and dry matter digestibility

Feeding NO_3^- + O decreased DMI relative to the O treatment ($P < 0.01$, Table 2). Compared with cattle fed the CON diet, feeding NO_3^- alone or in combination with canola oil reduced DMD by between 4 and 6 g/100 g DM respectively ($P < 0.01$).

3.2. Methane production and rumen fermentation

The CH_4-mitigating effect of NO_3^- and canola oil in combination (Nitrate × Oil, $P = 0.12$; Table 2) was synergistic. Feeding NO_3^- + O diet, reduced MY relative to cattle fed the other diets ($P < 0.01$) and no differences were observed between CON, NO_3^- and O treatments ($P > 0.05$). There was a tendency towards a positive correlation between DMD and MY in the 3 experimental periods for NO_3^- + O treatment (DMD = 0.53 + 0.01 MY, $r = 0.99$, $P = 0.09$). The dietary effect on CH_4 kinetics in an hourly basis is presented in Fig. 1. For the CON and the O diets 2 peaks of CH_4 production were observed at around 2 h after feeding. In contrast, with the NO_3^- and NO_3^- + O diets the peak in CH_4 production after feeding was less substantial.

Mean ruminal pH for the dietary treatments ranged from 5.98 to 6.30. The O treatment significantly reduced rumen pH compared to NO_3^- or NO_3^- + O treatments ($P < 0.01$). Nitrate and O-containing diets increased rumen NH_3-N concentrations ($P < 0.01$; $P = 0.06$) relative to CON.

Table 2
Rumen fermentation and physiological characteristics of cattle on a mixed lucerne/barley diet (CON) with or without supplements of nitrate (NO_3^-), canola oil (O) or NO_3^- plus canola oil (NO_3^- + O).

	Diet[1]					*P*-value[2]		
Parameter	CON	NO_3^-	O	NO_3^- + O	SEM	Nitrate	Oil	Nitrate × Oil
Final LW (kg)	715	711	712	705	4.78	0.21	0.30	0.74
DMI (kg/day)	6.64^{ab}	6.58^{ab}	6.86^{a}	6.36^{b}	0.05	< 0.01	0.86	0.02
DMD (g/kg DM)	731^{a}	688^{b}	721^{a}	674^{b}	2.98	< 0.01	0.60	0.02
MY (g CH_4/kg DMI)	23.2^{a}	21.2^{a}	21.8^{a}	17.4^{b}	0.62	< 0.01	< 0.01	0.09
DMP (g CH_4/d)	159.6	141.7	151.1	118.4	4.40	< 0.01	< 0.01	0.12
Rumen pH	6.2^{ab}	6.3^{a}	6.0^{b}	6.3^{a}	0.03	< 0.01	0.17	0.04
Rumen ammonia (mgN/L)	121.8	166.1	127	196	6.46	< 0.01	0.06	0.31
Total VFA (mmol/L)	104.2	99.5	106.5	100.1	1.61	0.20	0.93	0.93
Acetate (mol/100 mol)	62.3	67.4	59.1	64.1	0.42	< 0.01	< 0.01	0.69
Propionate (mol/100 mol)	16.1^{b}	15.2^{b}	19.1^{a}	15.8^{b}	0.28	< 0.01	< 0.01	0.03
Butyrate (mol/100 mol)	17.8^{a}	13.6^{c}	17.1^{ab}	16.2^{b}	0.33	< 0.01	0.09	< 0.01
Acetate:propionate	4.2	4.9	3.2	4.1	0.09	< 0.01	< 0.01	0.20
Total protozoa (log_{10}/ml)	6.3	5.6	5.9	6.0	0.36	0.02	0.01	0.95
Small entodiniomorph	6.3	5.6	5.9	5.9	0.40	0.03	0.02	0.82
Large entodiniomorph	4.7	2.5	4.5	3.7	0.34	0.01	0.23	0.41
Small isotrich	4.5	1.6	3.4	1.7	0.09	< 0.01	0.26	0.33
Large isotrich	2.0	1.1	2.0	2.0	0.09	0.40	0.39	0.36

[a,b,c]Mean values within a row with unlike superscript letters were significantly different for each dietary treatment (P < 0.05).
LW, live weight; DMI, DM intake; MY, methane yield; DMP, daily methane production; VFA, volatile fatty acid.
[1] CON = control; = NO_3^- = CON + 20 g NO_3^-/kg as-fed basis; O = CON + 50 g canola oil/kg as-fed basis; NO_3^- + O CON plus 20 g NO_3^-/kg as-fed basis and 50 g canola oil/kg as-fed basis.
[2] Nitrate represents the main effect of Nitrate (CON and O versus NO_3^- and NO_3^- + O); Oil represents the main effect of Oil (CON and NO_3^- versus O and NO_3^- + O); Nitrate × Oil represents the interaction between main effects of Nitrate and Oil.

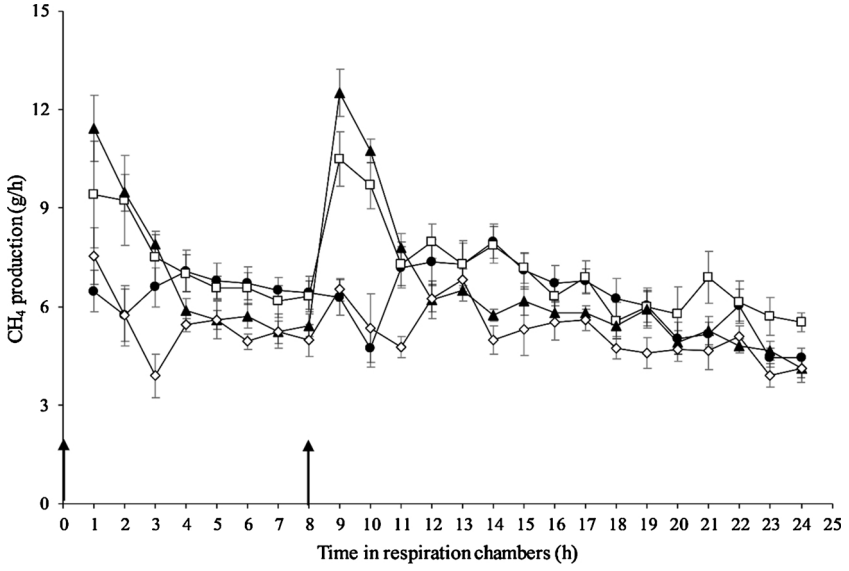

Fig. 1. Daily CH_4 kinetics in beef cattle fed diets supplemented with nitrate (NO_3^-) and canola oil alone or in combination. Vertical bars indicate SEM. Dietary treatments consisted of a control diet (CON, □), the CON diet with the inclusion of 20 g NO_3^-/kg as-fed basis (NO_3^-, ●), the CON diet with 50 g canola oil/kg as-fed basis (O, ▲), and the CON diet with 20 g NO_3^-/kg as-fed basis and 50 g canola oil/kg as-fed basis (NO_3^- + O, ◊). The arrows indicate time of feeding.

Concentrations of total VFA in the rumen did not differ between dietary treatments (P > 0.05; Table 2). However, NO_3^- inclusion increased acetate proportion (*P* < 0.01) and acetate to propionate ratio (A:P; P < 0.01); whereas these parameters were reduced by feeding the O diet. Propionate proportion was greater in cattle fed the O diet compared with the other diets (P < 0.01) whereas butyrate was greater in the O treatment relative to NO_3-fed cattle (P < 0.01). Feeding the NO_3^- diet significantly reduced butyrate proportion compared to the other dietary treatments (P < 0.01). Across all diets, small entodiniomorphs accounted for more than 90% of total protozoa present. Total rumen protozoa and small entodiniomorphs concentrations were decreased by including NO_3^- or canola oil, alone or in combination, in the diet of cattle (P < 0.05; Table 2). Feeding NO_3^- or NO_3^- + O diets reduced rumen

Table 3
Rumen digesta and microbial dynamics of beef cattle fed a lucerne chaff/barley diet (CON) or supplemented with nitrate (NO_3^-), canola oil (O) or NO_3^- plus canola oil (NO_3^- + O).

	Diet[1]					*P*-value[2]		
Parameter	CON	NO_3^-	O	NO_3^- + O	SEM	Nitrate	Oil	Nitrate × Oil
DM rumen digesta (g/kg)	120[b]	107[c]	137[a]	131[ab]	1.90	< 0.01	0.28	< 0.01
Rumen fluid volume (l)	68.7	87.1	34.3	43.5	6.99	0.24	< 0.01	0.42
Rumen fluid outflow rate (l/d)	54.7	78.1	48.3	69.0	5.22	0.05	0.28	0.50
Rumen DM pool size (kg)	11.2	7.3	5.1	4.3	1.11	0.35	< 0.01	0.19
Rumen DM outflow rate (kg/d)	6.5	7.6	6.0	5.7	0.63	0.04	< 0.01	0.70
Rumen fluid MRT (h)	34	27.7	16.9	15.3	3.00	0.57	< 0.01	0.44
Rumen particulate MRT (h)	43.4	22.6	20.6	14.7	3.88	0.03	< 0.01	0.17
Daily N intake (g N/d)	152[b]	174[a]	152[b]	177[a]	0.87	< 0.01	0.36	0.04
Pool size of rumen NH_3-N (mmoles)	585	877	315	623	31.8	< 0.01	< 0.01	0.42
ILR of ruminal NH_3-N (g N/d)	165	220	149	200	13.3	0.06	0.52	0.11
Proportion of microbial N from rumen NH_3[3]	0.8	1.0	0.9	0.8	0.06	0.84	0.10	0.14
Microbial growth efficiency (g MicNAN/kg DOMI)	11.8	8.2	15.4	9.8	1.79	0.28	0.55	0.81

[a,b]Mean values within a row with unlike superscript letters were significantly different for each dietary treatment ($P < 0.05$).
MRT, mean retention time; N, nitrogen; ILR, irreversible loss rate; NH_3, ammonia; MicNAN, microbial non-ammonia N; DOMI, digestible organic matter intake.
[1] CON = control; NO_3^- = CON + 20 g NO_3^-/kg as-fed basis; O = CON + 50 g canola oil/kg as-fed basis; NO_3^- + O CON with 20 g NO_3^-/kg as-fed basis and 50 g canola oil/kg as-fed basis.
[2] Nitrate represents the main effect of Nitrate (CON and O versus NO_3^- and NO_3^- + O); Oil represents the main effect of Oil (CON and NO_3^- versus O and NO_3^- + O); Nitrate × Oil represents the interaction between main effects of Nitrate and Oil.
[3] Calculated as Enrichment of ^{15}N in rumen bacteria/ Enrichment of ^{15}N in rumen ammonia.

concentrations of large entodiniomorphs ($P = 0.01$) and small isotrich ($P < 0.01$).

3.3. Chemical parameters and ammonia-N kinetics in the rumen

Feeding NO_3^- or NO_3^- + O diets increased daily N intake in cattle ($P < 0.01$) compared to the other diets; and so increased the pool size of ruminal NH_3-N ($P < 0.01$) and tended to have greater ILR of ruminal NH_3-N ($P = 0.06$). Adding NO_3^- alone to the diet of cattle reduced the DM content of rumen digesta compared with the other dietary treatments ($P < 0.05$; Table 3) whereas no effect was observed for NO_3^- + O treatment relative to CON ($P = 0.12$) and O diets ($P = 0.65$). Regarding rumen fluid, a greater rumen fluid outflow rate was observed in NO_3^--containing diets ($P = 0.05$) but rumen fluid MRT was not affected by NO_3^- ($P = 0.57$). Regarding rumen particles and DM, feeding NO_3^- increased rumen DM outflow rate ($P = 0.04$). An additive effect between NO_3^- and canola oil (Nitrate x Oil, $P = 0.17$) on reducing rumen particulate MRT ($P = 0.03$) was observed.

Feeding O-containing diets to cattle reduced the volume and MRT of the rumen fluid ($P < 0.01$), as well as the pool size ($P < 0.01$) and outflow rate of DM ($P < 0.01$) from the rumen. Supplementation with O reduced the pool size of rumen NH_3-N ($P < 0.01$) and tended to reduce the proportion of microbial N derived from ruminal NH_3-N ($P = 0.10$). There was no effect of the dietary treatment on microbial growth efficiency ($P > 0.05$; Table 3).

Table 4
Rumen flow of digesta and NAN as influenced by dietary treatment (control: CON; nitrate; NO_3^-; canola oil: O and NO_3^- plus canola oil: NO_3^- + O).

	Diet[1]					*P*-value[2]		
	CON	NO_3^-	O	NO_3^- + O	SEM	Nitrate	Oil	Nitrate × Oil
Flow of TD (kg/d)	14.3	12.8	10.0	10.8	0.42	0.68	0.02	0.22
Flow of DM (kg/d)	2.0	2.0	3.3	2.5	0.34	0.43	0.38	0.43
DMADR (kg)	3.6	4.3	3.5	4.1	0.33	0.40	0.95	0.91
NAN flow, (g N/d)	64.4	58.3	72.5	54.7	5.40	0.17	0.95	0.45
Proportion of microbial NAN in TD[3]	0.58	0.52	0.62	0.58	0.04	0.26	0.58	0.26
Flow of microbial NAN from the rumen (g micNAN/d)	36.4	30.4	40.1	31.2	2.30	0.10	0.58	0.75

NAN, non-ammonia nitrogen; TD, true digesta; DMADR, DM apparently digested in the rumen; N, nitrogen.
[1] CON = control; NO_3^- = CON + 20 g NO_3^-/kg as-fed basis; O = CON + 50 g canola oil/kg as-fed basis; NO_3^- + O CON with 20 g NO_3^-/kg as-fed basis and 50 g canola oil/kg as-fed basis.
[2] Nitrate represents the main effect of Nitrate (CON and O versus NO_3^- and NO_3^- + O); Oil represents the main effect of Oil (CON and NO_3^- versus O and NO_3^- + O); Nitrate × Oil represents the interaction between main effects of Nitrate and Oil.
[3] Calculated as Enrichment of ^{15}NAN in rumen TD/ Enrichment of ^{15}NAN in rumen microbes.

3.4. Rumen digesta flow

Flow of TD was less in cattle fed O-containing diets ($P < 0.05$; Table 4). However, no significant effects of the dietary treatments were observed for flow of DM, NAN or microbial NAN from the rumen of cattle ($P > 0.05$). There was also no effect on the proportion of NAN leaving the rumen that was of microbial origin and no treatment differences in the mass of DMADR ($P > 0.05$).

4. Discussion

The main objective of this experiment was to evaluate the effect of feeding NO_3^- and canola oil, alone or in combination, on enteric CH_4 production and nutrient supply to beef cattle, especially microbial NAN supply. It was shown that feeding NO_3^- and canola oil in combination are synergistic in reducing methanogenesis from cattle. There was, however, a negative effect of $NO_3^- + O$ diet on DMD which may have contributed to the reduction in MY through reducing substrate availability as did the slight reduction in DMI.

4.1. Daily methane production and methane yield

The antimethanogenic effect of NO_3^- has been well documented (Lee and Beauchemin, 2014). However, adding 20 g of NO_3^-/kg feed in this experiment had a less than expected effect on DMP. Assuming VFA proportions are unchanged, 1 mol of NO_3^- can be expected to reduce methanogenesis by 1 mol, so the inclusion of 20 g of NO_3^-/kg feed should have reduced methanogenesis by 0.323 mol or 5.17 g/kg DMI, however, the observed mitigation was only 39% of that expected. The less than expected CH_4 reduction may have been related to more H_2 availability in the rumen of NO_3^--fed cattle, as evidenced in the greater acetate proportion in the VFA. In addition, as shown in Fig. 1, the antimethanogenic effect of NO_3^- was sustained for less than 2 h after feeding the NO_3^- diet which is consistent with NO_3^- being rapidly metabolized in the rumen within 4 h after ingestion (Latham et al., 2016). A similar markedly effect of NO_3^- on CH_4 production immediately after feeding was also observed by Nolan et al. (2010) in sheep and Guyader et al. (2015) in dairy cattle. It may be that the less than expected mitigation of CH_4 by NO_3^- also resulted from incomplete NO_3^- reduction, due to absorption of NO_3^- and NO_2^- from the rumen prior to their reduction (Pfander et al., 1957) or flow from the rumen in digesta. At 44–87 L of liquid outflow from the rumen/d, ruminal outflow could have removed by about 13% of dietary NO_3^- unreduced, had rumen NO_3^- concentrations being approximately 40 ppm as found in sheep fed 2% NO_3^- in DM (de Raphélis-Soissan et al., 2016). It is not known if oil directly inhibits NO_3^- reduction, this may also have contributed to the lower than expected efficacy of NO_3^- (in association with oil) in mitigating DMP. In summary, while NO_3^- was effective in mitigating DMP, it is hypothesised that the less than expected CH_4 mitigation was a consequence of a number of small changes in VFA balance, and the absorption and outflow of NO_3^- and NO_2^- that together reduced the availability of both H_2 and NO_3^- and NO_2^- in the rumen, so reducing the efficacy of CH_4 mitigation by dietary NO_3^-.

In this experiment, feeding NO_3^- alone or in combination with canola oil reduced total protozoa concentration in the rumen. However, effects of NO_3^- on ruminal protozoa are contrasting in the literature. Nitrate alone was shown to reduce the number of protozoa in the rumen of sheep and goats supplemented with NO_3^- (Sar et al., 2005; Asanuma et al., 2015) but this effect was not always observed (Nolan et al., 2010; Guyader et al., 2015). Similarly, contradictory results have been reported with the addition of NO_3^- in combination with lipids on rumen protozoa concentration. Some authors reported NO_3^- and lipids having toxic effects on rumen protozoa (Morgavi et al., 2010) whereas others report no significant effect even in a long-term supplementation period (Guyader et al., 2016).

The antiprotozoal effect of canola oil observed in this experiment has also been reported previously (Tesfa, 1993). Poly-unsaturated fatty acids present in canola oil have a toxic effect on rumen microorganisms involved in H_2 production as ciliate protozoa (Doreau and Ferlay, 1995) and a reduction in rumen protozoa numbers is usually associated with a decrease in CH_4 production (Guyader et al., 2014). However, feeding the O diet alone in this experiment had no effect on DMP or MY. This result is in agreement with Duthie et al. (2016) testing rapeseed meal and rapeseed cake in cattle. The CH_4 suppressing effect of dietary oils has been inconsistent, with Grainger and Beauchemin (2011) reporting that lipids reduced MY by approximately 2.4% per 10 g/kg DM fat added to the diet and Beauchemin et al. (2008) reporting 5.6% CH_4 reduction for each 10 g of dietary fat/kg DM. A intermediate inhibitory effect was reported by Moate et al. (2011) who identified a 3.5% of CH_4 reduction per each 10 g/kg DMI of fat.

Our findings suggest a synergistic effect of feeding the $NO_3^- + O$ diet on reducing methanogenesis from beef cattle. The 25% reduction in DMD observed in $NO_3^- + O$ was in line with the findings of Guyader et al. (2015) and Popova et al. (2017). In combination, $NO_3^- + O$ caused a 25% reduction in MY which is consistent with lipids and NO_3^- having different and complementary mechanisms for supressing methanogenesis. Both additives included at the same time in the diet of ruminants attempt to reduce H_2 availability in the rumen, the main substrate for enteric CH_4 production (Hegarty and Gerdes, 1999). While oils rich in poly-unsaturated fatty acids have a toxic effect on H_2 producing protozoa and methanogens (Morgavi et al., 2010; Latham et al., 2016), NO_3^- reduction to NO_2^- and NH_3 in the rumen is energetically more favourable than the reduction of CO_2 to CH_4 (Jones, 1972).

Nitrate supplementation in ruminants has not been widely practiced due to the risk of NO_2^- toxicity. Feeding NO_3^- in combination with lipids, and including an adaptation period to the diet as in the present experiment, appears to be a practical strategy that should enable the livestock sector to mitigate enteric CH_4 emissions. In particular, using this $NO_3^- + O$ combination strategy could be a way of reducing the amounts of NO_3^- in the diet of ruminants, to achieve a mitigation target avoiding adverse effects on animal health.

4.2. Dry matter digestibility

The negative effect on DMD, of feeding NO_3^- as observed in this experiment is in agreement with Marais et al. (1988), although this has not always been reported (Olijhoek et al., 2016). The DMD reduction probably resulted from NO_2^- having a direct toxic effect on rumen microbes, which alters the microbial population in cattle fed NO_3^--containing diets. (Zhou et al., 2012).

A reduction in DMD has also been one of the most common findings associated with lipid supplementation in ruminants (Beauchemin and McGinn, 2006). Nevertheless, the lack of effect of feeding O on DMD observed in this experiment was in accordance with Brask et al. (2013), in dairy cows and may be related to the low inclusion of canola oil in the diet (50 g/kg DM). Patra (2013) suggested that when concentrations of lipids are below 70 g/kg DM, the lipids may not affect digestibility.

4.3. Rumen fermentation

Rumen fermentation as judged by total VFA concentration was not affected by dietary treatments in the current experiment. The greater proportion of butyrate detected in cattle fed the CON diet may be explained by the high number of protozoa in the rumen fluid which preferentially ferment OM to butyrate rather than acetate and propionate (Li et al., 2012). Nitrate supplementation in the present experiment shifted rumen fermentation towards acetate and increased A:P ratio, concurring with previous observations (Nolan et al., 2010). Nitrate has higher affinity for H_2 than does CO_2 and the reactions that generate propionate (Ungerfeld and Kohn, 2006) competing with CH_4 and propionate production.

In accordance with Machmüller et al. (2000) and Patra (2013), the proportion of acetate in VFA was unaffected by O inclusion in this experiment, but propionate percentage was significantly increased and so a reduction in A:P was observed. Adding canola oil to the diet of cattle may increase propionate proportion and reduce A:P (Pinares-Patiño et al., 2016), resulting in less H_2 availability in the rumen for CH_4 production, due to the net H_2 consumption in propionate synthesis (Newbold et al., 2015).

Although feeding the $NO_3^- + O$ diet significantly reduced rumen protozoa concentration in the present experiment, total VFA concentration and molar proportion of propionate were not affected. Previous experiments testing linseed oil in combination with NO_3^- reported no changes on total VFA and propionate proportion in the rumen of cattle (Guyader et al., 2015; Popova et al., 2017), although a toxic effect of linseed plus NO_3^- towards rumen protozoa was not observed. Protozoa effects on molar proportions of VFA are not consistent in the literature. Some reviews have reported an increase in molar proportion of propionate after defaunation (Hegarty, 1999; Eugène et al., 2004) whereas Newbold et al. (2015) showed no changes in total VFA concentration or in the molar proportion of propionate after removal of protozoa from the rumen. This diversity is likely to reflect the metabolic capability of the biota that refill the physical space otherwise occupied by protozoa.

4.4. Rumen ammonia and microbial nitrogen outflow

In keeping with NO_3^- reduction to NO_2^- and NH_3 in the rumen, ruminal NH_3-N concentration was increased by NO_3^- supplementation (NO_3^- and $NO_3^- + O$ treatments) which is in agreement with Hulshof et al. (2012). Rumen NH_3-N tended to be lost irreversibly at a greater rate in cattle fed NO_3^--containing diets but no effects were observed on microbial growth efficiency (g MicNAN/kg DOMI) with all values at the lower end of reported ranges (Poppi and McLennan, 2010). Feeding NO_3^- has been shown to increase microbial N outflow in lambs fed a protein-deficient chaff (Nguyen et al., 2016) and improved NH_3 incorporation into microbial protein in dairy cattle fed a low-protein diet (Wang et al., 2018). These results suggest that NO_3^- inclusion in the diet of ruminants may be more beneficial when dietary N is limiting.

In this experiment, the concentration of NH_3-N in the rumen was slightly increased in the O-containing diets in keeping with the significant reduction in the rumen protozoal population from the O treatment but in contrast with Beauchemin and McGinn (2006). In the same way, the significant decrease in ruminal protozoal population in cattle fed O-containing diets is normally associated with increased microbial NAN outflow from the rumen (Ivan et al., 1991). Removal of protozoa from the rumen reduces protozoa engulfment of bacteria while increasing microbial growth and duodenal N outflow (Eugène et al., 2004). However, no effects of the dietary treatment were observed in total and microbial NAN outflows in this experiment. Clearly, there are other factors affecting microbial NAN outflow and the lack of effect may have been a consequence of a smaller total rumen volume and hence total bacterial population in O-supplemented cattle.

The efficiency of microbial N synthesis is related to feeding level because higher DMI may increase ruminal passage rate of DM and the outflow of microbial protein (Barnett et al., 2012). In this experiment, O-containing diets increased DMI and decreased rumen MRT but did not affect microbial growth in the rumen of cattle. The lack of effect of lipid supplementation on microbial growth (g MicNAN/kg DOMI) in this experiment has also been reported by Leupp et al. (2006).

4.5. Digesta kinetics

Methane yield is positively associated with DMD, MRT and rumen volume (Hegarty, 2004; Pinares-Patiño et al., 2011a; Goopy et al., 2014) and this was observed for MY and DMD in cattle fed the $NO_3^- + O$ diet in this experiment. The increase in the MY with a longer rumen MRT is probably caused by increasing the extent of digestion of structural carbohydrates (Pinares-Patiño et al., 2011b). Although rumen particulate MRT was significantly decreased in all dietary treatments relative to CON, DMD was only reduced by feeding NO_3^--containing diets. This effect could be explained as compensatory DMD in the hindgut of cattle fed the O diet, as feeding oils to ruminants have been shown to shift the site of digestion from the rumen to the intestines (Ikwuegbu and Sutton, 1982). The

reduced time for rumen fermentation to occur as evidenced in the negative main effects of NO_3^- and O on rumen particulate MRT may have contributed to their lower MY. However, the direct antimicrobial effect of canola oil and the thermodynamic favourability of NO_3^- reduction would have had larger roles than MRT in suppressing CH_4 production.

5. Conclusions

This experiment shows that feeding dietary NO_3^- and canola oil in combination has a synergistic effect on reducing DMP and MY from beef cattle. The combination of both dietary additives will allow high levels of CH_4 mitigation to be sustained with a lower dose of NO_3^- and therefore a reduced likelihood of NO_2^- toxicity, which is a practical constraint to inclusion of NO_3^- in diets as a non-protein N source in ruminants. Reducing methanogenesis by the NO_3^- + O diet in this experiment did not improve microbial growth efficiency, the flow of MicNAN from the rumen or the utilization of rumen NH_3 for microbial growth, suggesting that this strategy seems to be more useful in N-limiting diets if production benefits are also been sought from these CH_4 suppressive additives.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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