Effects of Rumen Protozoa of Brahman Heifers and Nitrate on Fermentation and *In vitro* Methane Production

S. H. Nguyen[1,2,*], L. Li[1], and R. S. Hegarty[1]

[1] School of Environmental and Rural Sciences, University of New England, Armidale, NSW 2351, Australia

ABSTRACT: Two experiments were conducted assessing the effects of presence or absence of rumen protozoa and dietary nitrate addition on rumen fermentation characteristics and *in vitro* methane production in Brahman heifers. The first experiment assessed changes in rumen fermentation pattern and *in vitro* methane production post-refaunation and the second experiment investigated whether addition of nitrate to the incubation would give rise to methane mitigation additional to that contributed by defaunation. Ten Brahman heifers were progressively adapted to a diet containing 4.5% coconut oil distillate for 18 d and then all heifers were defaunated using sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol). After 15 d, the heifers were given a second dose of Empicol. Fifteen days after the second dosing, all heifers were allocated to defaunated or refaunated groups by stratified randomisation, and the experiment commenced (d 0). On d 0, an oral dose of rumen fluid collected from unrelated faunated cattle was used to inoculate 5 heifers and form a refaunated group so that the effects of re-establishment of protozoa on fermentation characteristics could be investigated. Samples of rumen fluid collected from each animal using oesophageal intubation before feeding on d 0, 7, 14, and 21 were incubated for *in vitro* methane production. On d 35, 2% nitrate (as $NaNO_3$) was included in *in vitro* incubations to test for additivity of nitrate and absence of protozoa effects on fermentation and methane production. It was concluded that increasing protozoal numbers were associated with increased methane production in refaunated heifers 7, 14, and 21 d after refaunation. Methane production rate was significantly higher from refaunated heifers than from defaunated heifers 35 d after refaunation. Concentration and proportions of major volatile fatty acids, however, were not affected by protozoal treatments. There is scope for further reducing methane output through combining defaunation and dietary nitrate as the addition of nitrate in the defaunated heifers resulted in 86% reduction in methane production *in vitro*. (**Key Words:** Defaunation, Refaunation, Nitrate, Fermentation and Methane Production)

INTRODUCTION

Reviews of the effects of enteric protozoa on digestion and productivity by ruminants have concluded removal of rumen ciliate protozoa reduces enteric methane (CH_4) emission by 13% (Newbold et al., 2015) and increases an average daily gain by 11% (Eugène et al., 2004). Finlay et al. (1994) concluded that methanogens existing as endo- and ecto-symbionts with ciliate protozoa contributed 37% of rumen CH_4 production and Stumm et al. (1982) identified that 10% to 20% of rumen methanogens were attached on the outside of protozoa. Centrifuging rumen fluid to remove protozoa reduced the methanogen population by 78% (Newbold et al., 1995).

Methane production is positively related to the size of the rumen protozoal population (Morgavi et al., 2010) and the absence of protozoa reduces CH_4 production and significantly modifies fermentation characteristics *in vitro* (Qin et al., 2012). However, Ranilla et al. (2007) reported that there was no correlation between methanogenesis and protozoal biomass per unit of feed degraded *in vitro*. Further, Bird et al. (2008) showed that defaunation did not change enteric CH_4 production 10 to 25 weeks post-treatment. Hegarty et al. (2008) also reported that rumen protozoa did not affect CH_4 production by lambs raised without protozoa from birth, or defaunated at weaning. Therefore, the role of protozoa in methanogenesis is unclear.

* Corresponding Author: S. H. Nguyen. Tel: +61-449169668, Fax: +61-267732362, E-mail: nghson.niah@gmail.com
[2] National Institute of Animal Sciences, Thuy Phuong, Tu Liem, Hanoi, Vietnam.

In contrast, dietary nitrate (NO_3) reduces CH_4 reliably and predictably (van Zijderveld et al., 2010; 2011). Nitrate reduces total gas production when rumen fluid is incubated *in vitro*, and it changes the volatile fatty acids (VFA) profile by increasing acetate and reducing propionate and butyrate molar proportions while total VFA concentration is unaffected (Lin et al., 2011).

The objectives of these studies were to describe the fermentation characteristics and CH_4 emission changes occurring in the period after refaunation of previously protozoa-free heifers, and assess whether NO_3 could further reduce CH_4 production from defaunated animals.

MATERIALS AND METHODS

Animals and feeding

All protocols for treatment and care of the cattle were approved by the University of New England Animal Ethics Committee (AEC 13-054). Ten Brahman heifers (8 months of age) with an average liveweight of 274±32.8 kg were used. Cattle were adapted to a pre-experimental diet of oaten (70%) and lucerne (30%) chaff with initial inclusion of 1% of coconut oil distillate (COD) which was raised to a final level of 4.5% over 8 d. Cattle were then changed to an experimental diet of for 10 d to eliminate rumen protozoa comprising oaten chaff (70%), lucerne chaff (21%), COD (4.5%) and molasses (4.5%), resulting in 88.1% dry matter (DM) in the mixed ration and 7.9% crude protein and 5% crude fat in the DM. This combined 18 d period of COD dietary treatment reduced the protozoal population from 3.91×10^5 cells/mL to 0.58×10^5 cells/mL of rumen fluid and all cattle were then treated with a chemical to defaunate. After the defaunation treatment, all cattle were given a diet of oaten (70%) and lucerne chaff (30%) which included 10.5% crude protein; 1.3 crude fat; 88.8% DM for the remainder of the study. All cattle had *ad libitum* access to the ration and water.

Defaunation of cattle

After 18 d feeding COD, all feed was withdrawn for a day and cattle were orally dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Allright and Wilson Australia Ltd, Melbourne, Australia) administered at 45 g/d in a 10% v/v solution to remove protozoa. Cattle were dosed on three consecutive days and feed was withheld during this treatment protocol, which was described by Bird and Light (2013). Animals required 15 d to fully recover their previous voluntary intake and received the COD diet during this period of time. The three day dosing with Empicol was then repeated commencing 15 d after the first dosing. A further 15 d after the second drenching program, rumen fluid samples were collected for protozoa enumeration and the experiment commenced (d 0).

Refaunation of cattle

On d 0 all cattle had recovered their intake and wellbeing, and rumen fluid of the animals was observed to be free of protozoa. Cattle were allocated to defaunated (n = 5) and refaunated groups (n = 5) by stratified randomisation based on liveweight. A single oral dose (500 mL/heifer) of a mixed rumen fluid collected from two cannulated faunated cattle grazing pasture was used to refaunate 5 heifers. The protozoal population in the inoculum (3.42×10^5 cells/mL) consisted of large holotrich (0.13×10^5 cells/mL), small holotrich (0.5×10^5 cells/mL) and small entodiniomorphs (2.79×10^5 cells/mL).

Rumen fluid sampling, ammonia, volatile fatty acid concentrations, and protozoal enumeration

In experiment 1, samples of rumen fluid (40 mL) were collected using oesophageal intubation from defaunated and refaunated heifers before feeding on d 0, 7, 14, and 21. Samples from defaunated heifers were immediately checked under a microscope to confirm that defaunated heifers were protozoa-free. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo Scientific, Beverly, MA, USA). A 15 mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and stored at –20°C for VFA and ammonia (NH_3) analyses. A 4 mL subsample was placed in wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoa were counted using a Fuchs-Rosenthal optic counting chamber (0.0625 mm^2 and 0.2 mm of depth) using a staining technique adapted from the procedure of Dehority (1984). The protozoa were differentiated into large (>100 μm) and small (<100 μm) holotrich and entodiniomorph groupings. Another 20 mL of subsample from defaunated and refaunated heifers was used to conduct *in vitro* incubations for methane measurements.

In experiment 2, samples of rumen fluid (~20 mL) were collected on d 35 using oesophageal intubation from defaunated and refaunated heifers before feeding, with each sample being processed individually and its incubation started immediately after collection. The VFA concentrations were determined by gas chromatography using a Varian CP 3800 Gas Chromatography (Varian Inc. Palo Alto, CA, USA) and NH_3 concentration was analysed using a modified Berthelot reaction using a continuous flow analyser (San^{++}, Skalar, Breda, The Netherlands).

In vitro incubations and measurements

In vitro incubations (23 h) were conducted using rumen fluid collected from defaunated and refaunated heifers on d 0, 7, 14, and 21 after refaunation, to assess changes in CH_4

production in defaunated and refaunated heifers, while rumen protozoa were re-establishing in refaunated heifers (experiment 1). Samples taken on d 35 were incubated *in vitro* with the addition of 2% NO_3 (as $NaNO_3$) of substrate DM to test for additivity of NO_3 and defaunation effects on fermentation and CH_4 production (experiment 2). The $NaNO_3$ was dissolved in purified water and added in buffer solution. The composition of incubation buffer was adapted and modified after Soliva and Hess (2007). For all *in vitro* incubations, 20 mL of rumen fluid from each animal was injected into a Schott bottle (100 mL) which contained 40 mL of buffer solution under a constant flow of anaerobic CO_2 in a water bath maintained at 39°C. Mixed rumen fluid and buffer solution (10 mL) was transferred into three 50 mL syringes (Luer lock: Terumo Corporation, Tokyo, Japan) which contained 200±20 mg of ground substrate (70% oaten and 30% lucerne chaff). The syringes were sealed by a 3-way tap, pre-warmed to 39°C and then incubated in a shaking water bath at 39°C. After the incubations, gas volume was measured, liquid was drained from the syringes and placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulphuric acid and stored at –20°C for VFA and NH_3 analyses. The gas in the syringes were analysed for CH_4 concentration using a Varian CP 4900 Gas Chromatography (Varian Inc., USA).

Statistical analyses

Data were statistically analysed using SAS 9.0 (SAS Inst., Cary, NC, USA). Data from experiment 1 were subject to repeated-measures analysis of variance in PROC MIXED with protozoa, time and protozoa×time interaction as fixed factors. Data from experiment 2 were subject to analysis of variance in PROC GLM, factors being protozoa, NO_3 and protozoa×NO_3 interaction. Means were analysed using the least squares means (LSMEANS) procedure. A probability of <5% was considered to be statistically
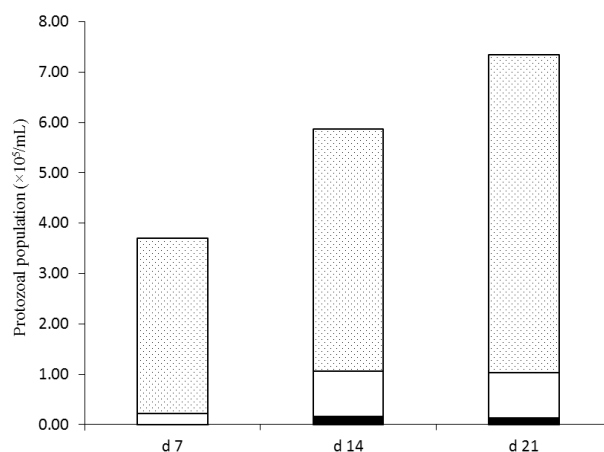
Figure 1. Small holotrich (□), large holotrich (■) and small entodiniomorphs (▦) from refaunated heifers 7, 14 and 21 d after refaunation.

significant.

RESULTS

Protozoal population in refaunated heifers

Protozoa were not observed in any rumen fluid samples collected from defaunated heifers during this study. In refaunated heifers, however, the protozoal population reached 3.70×10^5 cells/mL by d 7 and almost doubled by d 21 (7.01×10^5 cells/mL). Small entodiniomorphs were predominant in the total population, accounting for 94%, 82%, and 86% of the total counts at d 7, 14, and 21, respectively (Figure 1). Methane production from refaunated heifers was positively correlated with protozoal numbers although CH_4 production tended to stabilise after d 14 (Figure 2).

***In vivo* fermentation pattern and *in vitro* methane**
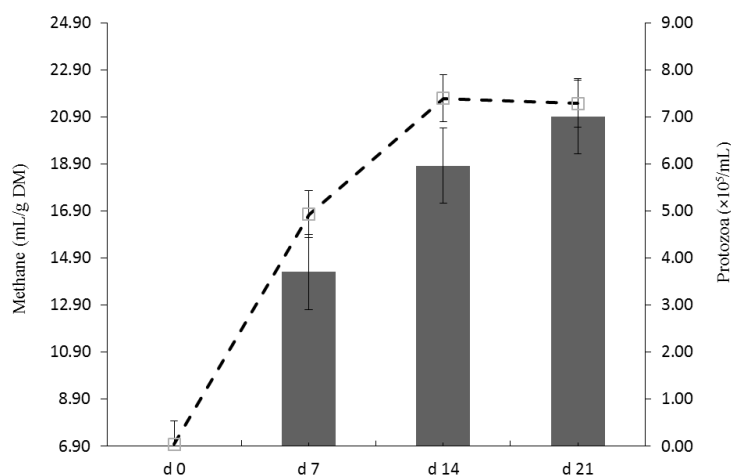
Figure 2. Methane production (□) and protozoal population (■) in rumen fluid from refaunated heifers 0, 7, 14, and 21 d after refaunation using a mixed rumen fluid inoculum. Error bars indicate standard error of the mean.

Table 1. The pH, ammonia concentration and concentration and molar proportions of major volatile fatty acids (VFA) in rumen fluid, and changes in gas and methane production *in-vitro* after refaunation

Item[1]	Treatment								SEM	p-values		
	Defaunated				Refaunated					Trt	Time	Trt×time
	Day 0	Day 7	Day 14	Day 21	Day 0	Day 7	Day 14	Day 21				
pH	6.41	6.46	6.87	6.83	6.62	6.69	6.86	6.91	0.10	0.02	<0.001	0.34
Ammonia (mg/L)	32.68	30.76	59.04	62.92	36.88	69.52	86.24	117.00	9.56	<0.01	<0.001	0.08
Total VFA(mM/L)	64.43	59.67	50.92	57.95	59.46	63.43	63.03	58.16	8.05	0.63	0.18	0.39
VFA molar proportion (%)												
Acetate (%)	71.06	74.55	75.32	79.01	73.67	73.49	73.39	76.74	1.76	0.59	0.04	0.51
Propionate (%)	19.15	16.61	15.05	14.46	17.75	15.66	14.52	12.30	1.40	0.12	0.02	0.95
Butyrate (%)	8.38	7.05	6.54	6.39	6.77	8.03	8.44	7.39	0.65	0.37	0.60	0.03
Acetate/propionate	4.07	4.65	5.08	5.57	4.58	4.77	5.07	6.29	0.51	0.26	0.44	0.90
Total gas[2] (mL/g DM)	102.33	128.67	144.07	157.00	103.67	135.67	152.00	149.33	4.71	0.55	<0.001	0.34
CH_4 (mL/g DM)	6.44	13.60	16.86	20.66	6.99	16.76	21.68	21.47	1.29	0.07	<0.001	0.19

SEM, standard error of the mean; Trt, treatment (defaunated and refaunated); DM, dry matter.

[1] pH, ammonia and VFA analyses on samples collected from animals on d 0, 7, 14, and 21.

[2] Gas and methane production data collected from *in-vitro* incubations.

production in experiment 1

The rumen fluid pH was higher (p = 0.02) in refaunated heifers, but increased (p<0.001) from d 0 to d 21 in both defaunated and refaunated heifers, showing effects of protozoal treatments and time (Table 1). Ammonia concentrations increased steadily up to d 7 in both defaunated and refaunated heifers, but refaunated heifers had higher NH_3 concentrations than did defaunated heifers (p<0.05). Neither VFA concentration, nor molar proportions of acetate, propionate and butyrate in total VFA, or acetate to propionate ratio were affected by protozoal treatment, but all except butyrate proportion increased over time.

There was an increase in total gas production *in vitro* by rumen fluid collected from both defaunated and refaunated heifers from d 0 to 14 with no significant further increase to d 21. There was a tendency towards a lower CH_4 production from rumen fluid of defaunated heifers than from refaunated heifers over time (p = 0.07). No significant interaction between protozoal treatment and time was observed (p>0.05).

***In vitro* fermentation pattern and methane production in experiment 2**

The pH after incubation was increased by the presence of protozoa and by NO_3 (Table 2). Ammonia concentration was also increased by protozoal treatments and by NO_3 (p<0.05). The presence of protozoa had little effect on VFA, with total VFA concentration tending to be lower in rumen fluid from defaunated than refaunated heifers, but VFA proportions were unaffected. VFA concentration was significantly reduced by NO_3 and a significant reduction in butyrate percentage also occurred.

Methane production was reduced by both defaunation and by NO_3, and there was a significant interaction between defaunation and NO_3 such that mitigation resulting from NO_3 and defaunation was greater than the mitigation

Table 2. The pH, ammonia concentration, volatile fatty acid (VFA) concentration and molar proportions and methane production as influenced by the presence or absence of protozoa (F, fauna) or nitrate (NO_3) addition for incubations of rumen fluid *in-vitro*

Item	Treatment				SEM	p-values		
	Defaunated		Refaunated			F	NO_3	F×NO_3
	-NO_3	+NO_3	-NO_3	+NO_3				
pH	6.19	6.49	6.02	6.32	0.05	<0.01	<0.01	0.98
Ammonia (mg/L)	101.19	185.71	167.23	211.70	11.60	0.01	0.01	0.18
Total VFA (mM/L)	102.96	83.15	137.57	98.01	12.14	0.08	0.04	0.14
VFA molar proportion (%)								
Acetate (%)	69.33	70.30	67.75	68.74	1.95	0.45	0.63	0.10
Propionate (%)	20.34	22.48	19.57	21.54	1.14	0.47	0.11	0.94
Butyrate (%)	9.57	6.78	10.96	8.49	1.10	0.20	0.04	0.89
Acetate/propionate	3.42	3.15	3.47	3.26	0.26	0.76	0.39	0.92
Total gas (mL/g DM)	155.00	101.67	149.44	117.78	4.14	0.21	<0.01	0.01
CH_4 (mL/g DM)	18.59	3.00	22.11	12.73	0.63	<0.01	<0.01	<0.01

SEM, standard error of the mean; F, fauna status; DM, dry matter.

resulting from either alone (p<0.05). Methane production from defaunated heifers was lower than from refaunated ones (18.59 vs 22.11 mL/g DM). While NO_3 reduced the CH_4 production in refaunated heifers (12.73 vs 22.11 mL/g DM), the combined effects of defaunation and dietary NO_3 on CH_4 mitigation (19.11 mL) was greater than the sum of effects of defaunation (3.52 mL) and NO_3 (9.38 mL), implying the combined treatments were more than additive in their mitigation potential. Total gas production was not affected by protozoal treatments (p>0.05), but was reduced in incubations containing NO_3 (p<0.05).

DISCUSSION

The objectives of this study were to describe the changes in CH_4 production and rumen fermentation characteristics associated with the reintroduction of protozoa into previously protozoa-free heifers and also assess whether CH_4 mitigation arising from NO_3 would be additive to that caused by the absence of protozoa. The protozoal population in previously defaunated heifers was established by d 7 and reached 7.01×10^5 cells/mL by d 21 comparable with that found by Morgavi et al. (2008) in sheep. These authors demonstrated that total protozoal population reached their peak at 12×10^5 cells/mL at 25 to 30 d after inoculation and then stabilised at 7.6×10^5 cells/mL from d 60. During the refaunation period there was a substantial increase in CH_4 production rate; this result was in accordance with the positive correlation between protozoa and CH_4 production found by Morgavi et al. (2010) and the fact methanogens that are normally attached to protozoa (Newbold et al., 1995) are responsible for 37% of rumen CH_4 emission (Finlay et al., 1994). The present study showed that rumen fluid from previously defaunated heifers tended to have lower CH_4 production *in vitro* than samples from refaunated heifers 7, 14, and 21 d after refaunation. This effect may not be exclusively a direct consequence of protozoa but also an indirect consequence of differences in bacterial and fungal populations in the presence of protozoa (Eugène et al., 2004) and in some cases, an increase in activity of H_2 producers (Morgavi et al., 2012). Such compensatory changes in microbial populations after defaunation leading to an unchanged VFA pattern may explain why the absence of protozoa has caused no significant changes in CH_4 emission in defaunated animals as observed from some previous studies (Bird et al., 2008; Hegarty et al., 2008; Morgavi et al., 2012).

Effects of protozoa on rumen NH_3 concentrations are generally more consistent than effects on VFA concentration with the concentration of NH_3 lower in defaunated ruminants compared to faunated or refaunated

ones in this and previous studies (Jouany et al., 1988; Eugène et al., 2004; Santra et al., 2007; Morgavi et al., 2012; Newbold et al., 2015). Defaunation has sometimes increased total VFA concentration in defaunated sheep (Santra et al., 2007) and weaner lambs (Santra and Karim, 2002), but Hegarty et al. (2008) found total VFA was lower and the proportion of propionate was reduced in the protozoa-free lambs born from defaunated ewes. These authors suggested that effects of defaunation on reducing CH_4 production may be dependent upon fermentation shifting to a more propionate rich pattern in defaunated animals. This is consistent with defaunation normally increasing the proportion of propionate and decreasing the proportion of butyrate while concomitantly reducing methane output (Eugène et al., 2004; Morgavi et al., 2012). No differences between defaunated and refaunated heifers in concentration and proportions of VFA were observed in these studies but the absence of rumen protozoa still reduced CH_4 production (experiment 2), indicating that protozoal effects on methanogenesis are not just a consequence of increased partitioning of H_2 into propionate synthesis.

Importantly, the successive *in vitro* studies showed that despite defaunation being completed 15 d before d 0; the rumen of defaunated heifers was not metabolically stable, with pH, total VFA, NH_3 and CH_4 production changing out to d 21 in the experiment 1. Little is known about rumen ecological stabilisation after defaunation, it was presumable in these studies that rumen ecology was stable within 50 d after defaunation and therefore was stable when the combined effects of NO_3 and defaunation were assessed in experiment 2.

Dietary NO_3 has been shown to offer a reliable and predictable strategy to mitigate CH_4 production from ruminants in both *in vitro* and *in vivo* studies. A review by Leng and Preston (2010) concluded that the use of NO_3 as a hydrogen sink could reduce CH_4 production from 16% to 50%, depending on diets and the inclusion rate of NO_3. This is because approximately 2 moles of hydrogen will be needed to convert NO_3 to nitrite and 6 moles hydrogen will be removed in order to reduce nitrite to NH_3 (Allison and Reddy, 1984). The result from the experiment 2 showed that CH_4 production was significantly lowered by addition of NO_3 in refaunated heifers 35 d after refaunation, confirming the potential for role of dietary NO_3 as a strategy to mitigate CH_4 emission (Guo et al., 2009; Nolan et al., 2010; van Zijderveld et al., 2010; 2011). In addition, NO_3 reduced total gas production, total VFA concentrations and the proportion of butyrate *in vitro* in line with findings of Lin et al. (2011). The present study also indicated that the combined effects of protozoal treatment and dietary NO_3 led to more than additive reduction in CH_4 production

(19.11 mL) compared with the sum of the protozoal effect (3.52 mL) and the dietary NO_3 effect (9.38 mL).

CONCLUSION

Methane production was positively correlated with protozoal numbers in rumen fluid in the period following refaunation of defaunated heifers with protozoa. The absence of protozoa reduced CH_4 production by 16% compared with refaunated heifers, dietary NO_3 reduced CH_4 production by 42% and the combined effects of NO_3 and defaunation reduced CH_4 production by 86%. Future research is needed to confirm these suggestions and gain better understandings the changes in gut fermentation, adaptation of methanogens and increased activity of some rumen microbes after defaunation and refaunation. *In vivo* trials need to be undertaken to gain a better understanding of the combined effects of defaunation and dietary NO_3 on CH_4 production in ruminants.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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