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IN VITRO INHIBITION OF METHANE PRODUCTION IN BOVINE RUMEN FLUID CULTURES BY RED LAKE EARTH, A NATURAL DIATOMACEOUS EARTH-CALCIUM BENTONITE BLEND

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In vitro inhibition of methane production in bovine rumen fluid cultures by Red Lake Earth, a natural diatomaceous earth-calcium bentonite blend

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CORRIE BELANGER

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This thesis has been accepted as conforming to the required standards by:

Jonathan Van Hamme (Ph.D.), Thesis Supervisor, Dept. Biological Sciences

John Church (Ph.D.) Co-supervisor, Dept. Natural Resource Sciences

Ron Smith (Ph.D.), Examining Committee member, Dept. Biological Sciences

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1 ABSTRACT

Farming ruminants contributes to over 25% of anthropogenic methane (CH_4), a compound which is 25 times stronger at heat trapping than CO_2, making it one of the most impactful of greenhouse gases. Because of this, mitigation of CH_4 emissions is critical both environmentally and agriculturally. In this study, the effects of an approved feed additive, Red Lake Earth (RLE), on cattle rumen fluid methane production was observed *in vitro*. Pure diatomaceous earth (DE), pure calcium bentonite, and mined RLE containing a mixture of these two materials, were tested for their effects on CH_4 production *in vitro* at concentrations from 20 to 100 g/L. After 24 hours of incubation, CH_4 production was measured directly from culture headspace using gas chromatography. RLE treatments were found to reduce CH_4 production by 90.5% ± 1.9 % compared to untreated cultures, and the 20 g/L treatment was selected for 5 day time course studies using rumen fluid from 4 independent animals. Methane production in RLE treated, 5-day cultures was, on average, inhibited by 59% ± 11% compared to untreated controls. Liquid culture samples were analyzed using quantitative real time PCR (qPCR; *mcrA*, 18S and 16S rDNA) and reverse transcriptase qPCR (*mcrA*) to quantify the effects of RLE on numbers of protozoa, bacteria and archaea in cultures. No significant changes in total archaea, bacteria, *mcrA* genes or protozoa were found when cultures were treated with RLE. However, there was a significant reduction in the transcript to gene ratio of the *mcrA* gene suggesting that RLE may inhibit methanogenesis by indirectly reducing transcriptional activity. This research strongly supports the ability of RLE to reduce rumen CH_4 production *in vitro* by reducing methanogen activity and suggests further genomic and *in vivo* studies to assess the potential impact of RLE as a feed additive.

Supervisor: Dr. Jon Van Hamme

Co-supervisor: Dr. John Church

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4 INTRODUCTION

Methane (CH_4) is a potent greenhouse gas, which is 25 times stronger at heat trapping than carbon dioxide (CO_2) (as reported by IPCC, 2007). In 2010, global anthropogenic CH_4 emissions were estimated to be around 7,800 million metric tons of CO_2 equivalents which is slightly more than $1/5^{th}$ of the amount of anthropogenic CO_2 emitted in the same year (as reported by IPCC, 2014). Studies have shown that, in 2010, approximately 29% of anthropogenic CH_4 was released by rumen microorganisms from farm animals such as cattle (as reported by IPCC, 2007; GMI, 2011).

The primary microorganisms involved in CH_4 production in the rumen are methanogens and ciliate protozoa. Methanogens are archaea that exist symbiotically with primary and secondary fermenters, and reduce CO_2 to CH_4 using H_2, produced from fermentation of feeds, as an electron donor (Morgavi et al., 2010). Research has suggested that rumen ciliate protozoa have a close relationship with methanogens in this process, and may provide direct transfer of H_2 (Beauchemin et al., 2011; Morgavi et al., 2010). Previous studies exploring treatments for mitigating rumen methanogenesis have included alteration of diet, use of vaccines and inhibitors, selective breeding, and defaunation (Beauchemin et al., 2011; Ferry, 2010). In a recent review, it has been suggested that diet manipulation is the strongest and most direct way of reducing CH_4 emissions from ruminants (Beauchemin et al., 2011).

Red Lake Earth (RLE) is a natural mixture of diatomaceous earth (DE) and calcium bentonite, materials that are used as anti-caking agents in animal feeds (Korunic, 1998; Bernard et al., 2009). DE has previously been implicated with the ability to reduce parasites in animals and

grain stocks, and may have an effect on animal nutritional status (Fernandez et al., 1998; Frost et al., 2007). Bentonite as a feed additive has been shown to improve the supply of feed and bacterial protein to the intestinal tract of sheep (Ivan et al., 1992). As a natural mixture of DE and bentonite, two feed additives used to improve parasite load and feed digestion in ruminants, RLE has potential to affect the microbial communities in rumen cultures *in vitro*.

In preliminary, unpublished research, RLE at 50 and 100 g/L powder granulations, significantly ($P = 0.01$ and $P = 0.0005$, respectively) inhibited *in vitro* methane production over the course of 5 days. In this paper, a thorough examination of the effects of various concentrations of RLE on CH_4 production and microbial communities *in vitro* was done to assess its potential CH_4 mitigation properties.

5 MATERIALS AND METHODS

5.1 Rumen fluid collection

Rumen fluid was collected from Kam Lake View Meats (Cherry Creek, BC, Canada) and Rainer Meats (Darfield, BC, Canada) from grass or hay fed cattle. Fluid was collected using nitrogen flushed glass jars preheated to 39°C from recently slaughtered cattle. The collected fluid was transported to the lab in an insulated container and used as an inoculant within an hour and a half of collection. One litre of the first rumen fluid collected was used to make clarified rumen fluid by filtering through four layers of cheese cloth, centrifuging at 3000 × *g* for 20 minutes, then autoclaving for 20 minutes at 827 kPa and 121 °C. The clarified fluid was allowed to cool to room temperature and stored at -20°C for future use (Kenters et al., 2011). For inoculation (2 mL inoculum per 27 mL liquid culture) into cultures, rumen fluid samples

were blended for 30 seconds to remove attached cells from particulate matter, and then strained through four layers of cheese cloth.

5.2 Media preparation and experimental design

Anaerobic growth medium was prepared according to Flythe and Aiken (2010), and contained, per litre: 240 mg Na_2HPO_4, 240 mg KH_2PO_4, 480 mg NaCl, 100 mg $MgSO_4$, 64 mg $CaCl_2$ H_2O, 600 mg cysteine·HCl and 4 g Na_2CO_3. The following additional components were included, per litre: 500 mg cellobiose (Alfa Aesar, Heysham, Lancaster, England; 98+ %), 13 mL of 0.1 g/L resazurin (an anaerobic indicator; Aldrich, St. Louis, MI, USA; 95%) in water, and 150 mL clarified rumen fluid

Rumen fluid cultures were set up in triplicate in 70-mL serum bottles (Wheaton Industries Inc., Millville, NJ) sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ) and aluminum crimp seals (Bellco Glass Inc., Vineland, NJ) to test the effects of RLE, pure DE and calcium bentonite, at concentrations of 20, 50, 75 and 100 g/L, on methane production. Controls contained no inoculum (NI), no additive (NA), or 2-bromoethanesulfonate (BES; 12 mM), a known inhibitor of methyl co-reductase M. Four independent experiments were completed, each using rumen fluid from a separate animal.

Following screening experiments, RLE at a concentration of 20 g/L was selected for time course analyses. Samples containing RLE, and BES, NA and NI controls, were set up with five replicates each. The cultures were incubated for 5 days with sampling times at 0, 12, 24, 48, 96, and 120 hours with the 0 hour samples for microbial analysis taken directly from the rumen fluid used as inoculum. Four independent experiments were completed with rumen fluid from separate animals.

5.3 CH_4 measurements

Headspace pressure was measured using a USB pressure transducer (Omega Engineering Inc., Laval, QC, Canada) with a 20-gauge needle (Mauricio et al., 1999). Pressure values were used to calibrate gas volumes to standard temperature and pressure (Table 1 in appendix).

For both screening and time course experiments, CH_4 was measured directly from the headspace on a gas chromatograph (GC) (Varian, model 3800; Agilent Technologies, Mississauga, ON, Canada) equipped with a flame ionization detector and a CP-1177 injector. A syringe equipped with a 26-gauge needle was used to anaerobically remove 0.2 mL of headspace gas for direct injection into the GC. A GS-Gaspro column (30 m × 0.32 mm, Agilent Technologies, Mississauga, ON, Canada) was used, with the injector and detector set to 225°C, the oven temperature at 60°C, and a column flow of 4.0 mL/min.

Methane standards of 2.5, 5, 10, 20, 50 and 100 mL/L were prepared using CH_4 (Praxair Canada Inc., Kamloops, BC, Canada; grade 3.7, 99.97% pure) injected in different volumes into 11-mL glass vials (Labco Lmt., Lampeter, Ceredigion, UK) (Nelson, 1971). A new standard curve was prepared daily, and the curves were found to be highly reproducible (See Figure 5 in appendix)

5.4 Microbial analysis

DNA and RNA were extracted in triplicate from controls, RLE, and BES-containing cultures during 5-day time course experiments. DNA was extracted using an Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), and RNA was extracted with an E.Z.N.A. Soil RNA Mini Kit (Omega Bio-Tek Inc., Norcross, GA, USA). cDNA was prepared from RNA using 5× qscript cDNA SuperMix (Quanta Biosciences Inc., Gaithersburg, MD,

USA) in a thermocycler (Bio-Rad S1000; Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) set for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. DNA concentrations were determined by fluorometric detection (Qubit 1.0 Fluorometer; Life Technologies Corp., Carlsbad, CA, USA)

Quantitative real time PCR (qPCR) was used to quantify total bacteria (16S rRNA gene), total protozoa (18S rRNA gene), and total methanogen methyl-coenzyme M reductase (*mcrA*) gene and transcript copy numbers using DNA and cDNA, respectively. Assays were performed using an Eco Real-Time PCR system (Illumina Inc., San Diego, CA, USA), and Platinum SYBR Green qPCR Supermix-UDG (Life Technologies Corp., Carlsbad, CA, USA). The primers for targeting 16S rRNA, 18S rRNA and *mcrA* genes were 519-f/ 915-r, 316f/ 539r, and qmcrA-f/ qmcrA-r, respectively (Denman et al., 2007; Sylvester et al., 2004; Jeyanathan et al., 2011; see Table 2 in the appendix). After optimization, a primer concentration of 0.3 μM and 2 ng DNA template was used for each assay. Template-free controls as well as no-reverse transcriptase controls were included with each run to ensure there was no DNA contamination. The temperature cycle was as follows: one cycle of 15 min at 95°C for initial denaturation, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 59°C (16S rRNA), 54°C (18S rRNA), or 60°C (*mcrA*), and 1 min at 75°C for primer annealing and elongation. Amplicon specificity was determined by melt curve analysis by increasing the temperature from 55°C to 95°C in 3 min.

Standard curves for the quantification of total bacteria were produced using 16S rRNA gene template from *Gordonia* sp. NB4-1Y. Standard curves for quantification of protozoa were prepared using amplified 18S rRNA template of rumen fluid protozoa samples. Standard curves for the quantification of methanogens were produced with *mcrA* gene template

amplified from *Methanobrevibacter ruminantium* DNA (DSMZ, Braunschweig, Germany). DNA was quantified using a Qubit Fluorometer, and 1:10 serial dilutions were performed to make standard curves from 2.5×10^8 copies to 250 copies per µL. Standard curves were prepared in triplicate for every qPCR quantitation, and were found to be highly reproducible (See Figure 6 in the appendix).

5.5 Statistical analysis

All statistical analyses were done in RStudio version 0.98.1103 (RStudio, Inc.).

Effects of treatments in methane screening experiments were tested for normality using the Shapiro test. Since all data was not determined to be normal, the Kruskal-Wallis test was performed to compare significance of variance between the means of each sample at each concentration. Kruskal-Wallis multiple comparisons test was used to determine significance of differences between individual treatments.

Effects of RLE treatments on methane production and microbial communities in time course cultures was assessed using mixed ANOVA. Shapiro-Wilks and Levene's tests were used to determine normality and homogeneity of the data set, respectively. Since neither data set was found to be homogenous, methane and qPCR data were square root and logarithm transformed, respectively. Simple ANOVAs were done to assess differences between treatments at individual time points.

6 RESULTS

6.1 CH_4 measurements

Methane was measured in screening experiments after 24 hours of incubation (Figure 1) in order to select a single concentration for time course studies. Since Kruskal-Wallis test showed that there was a significant difference between treatments but not a significant difference between subjects, the data was averaged between animals. Cultures containing RLE inhibited CH_4 production by 90.7% ± 1.9%, 95.0% ±2.1%, 96.6% ± 1.3%, and 97.5% ± 0.5% in cultures treated with 20, 50, 75, and 100 g/L, respectively ($P<0.001$) compared to cultures not treated with any substrate. Pure DE showed CH_4 production was only significantly inhibited by 81.2% ± 7.8% and 69.2% ± 16.9% at the highest concentrations of 75 and 100 g/L, respectively ($P=0.003$ for both). Calcium bentonite, when added at the concentrations used, absorbed all of the liquid in the cultures and was not used in subsequent experiments. RLE at 20 g/L reduced methane production by an average of 90.7% ± 1.9% ($P=0.0004$) compared to no-additive controls. RLE at 100 g/L reduced methane production by an average of 97.5% ± 0.5% ($P=0.0001$).

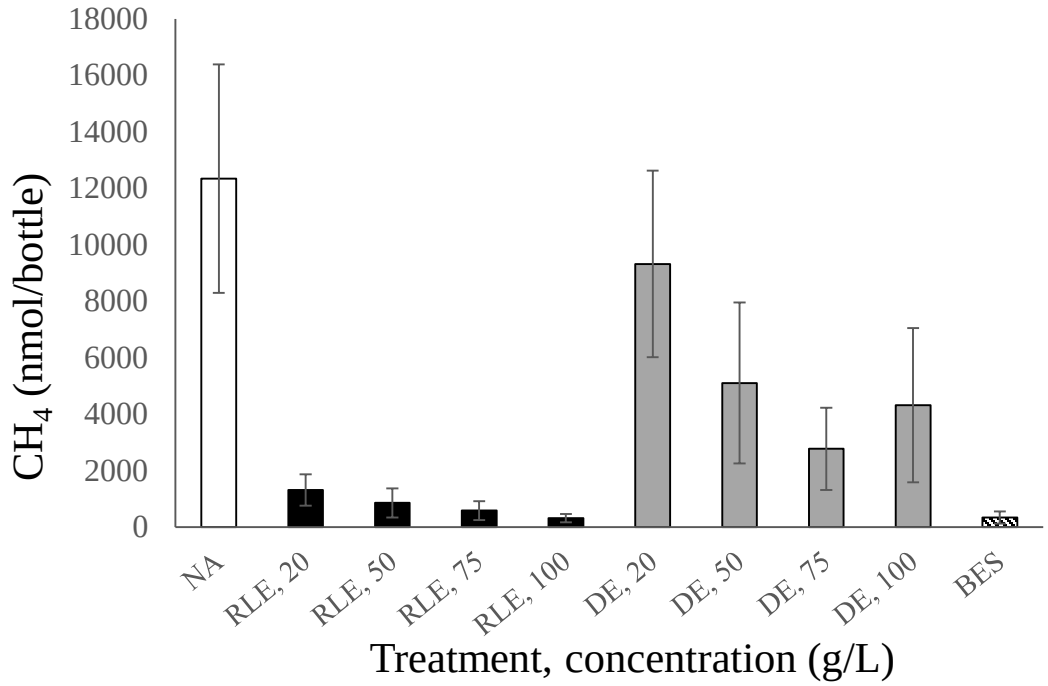

Figure 1. CH_4 production in rumen fluid cultures treated with RLE (Black; $P<0.001$), DE (grey; $P=0.003$ for 75 and 100 g/L) and BES (diagonal stripes; $P<0.001$), compared to NA controls (white). Error bars represent standard errors from 4 independent experiments, n=3 for each treatment in each experiment. CH_4 was measured by GC directly from headspace of anaerobic rumen fluid cultures incubated for 24 hours at 39°C.

The lowest concentration of RLE (20g/L) was chosen for further time course studies examining CH_4 production and bulk changes in microbial communities at six time points over a five day period. NA and BES controls were included. The RLE treatment reduced methane production compared to the NA control in 4 independent experiments and for all time points after 0 h ($P \leq 0.001$) (Figure 2). Overall, methane production after five days was on average of 59% ± 11.3% less in cultures treated with RLE compared to the NA cultures.

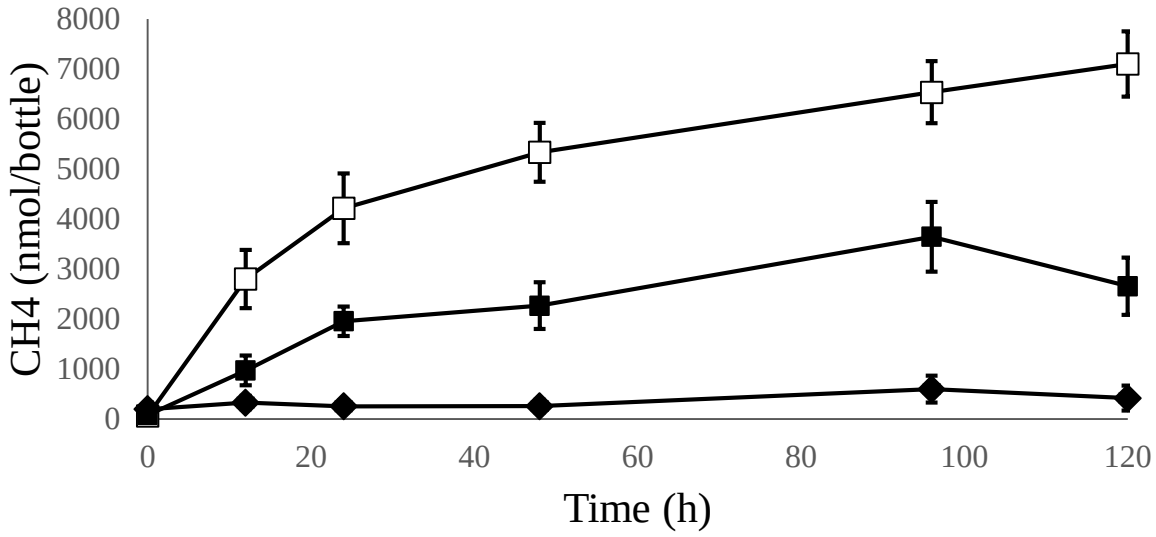

Figure 2. Time course of CH_4 production in rumen fluid cultures treated with RLE (■) ($P<0.001$) compared to no additive control (NA; □). BES (◆), a known inhibitor of methanogenesis, was used as a positive control. CH_4 was measured directly from anaerobic rumen fluid cultures incubated for 120 hours at 39 °C. Error bars represent standard error from 4 independent experiments, n=5 for each treatment in each experiment.

6.2 Microbial community effects

In order to monitor bulk shifts in the numbers of prokaroyotes (bacteria, archaea), protozoa and methanogenic archaea, DNA extracted from replicate cultures from the time course experiment was pooled for each animal and diluted to normalize the amount of DNA being used for qPCR analysis. Pooled samples representing the independent experiments established from 4 different animals (n = 4) were analyzed in duplicate and each standard was analyzed in triplicate for every qPCR assay. Quantities of bacteria/archaea, protozoa, and mcrA genes and transcripts were calculated from a new standard curve for each qPCR assay. R^2 values for standard curves were all 0.99 or greater, and efficiency values ranged from 80% to 130%. Total bacteria and archaea, as well as methanogens and protozoa, appeared not to be affected by the RLE treatment after 120 hours (Figure 3).

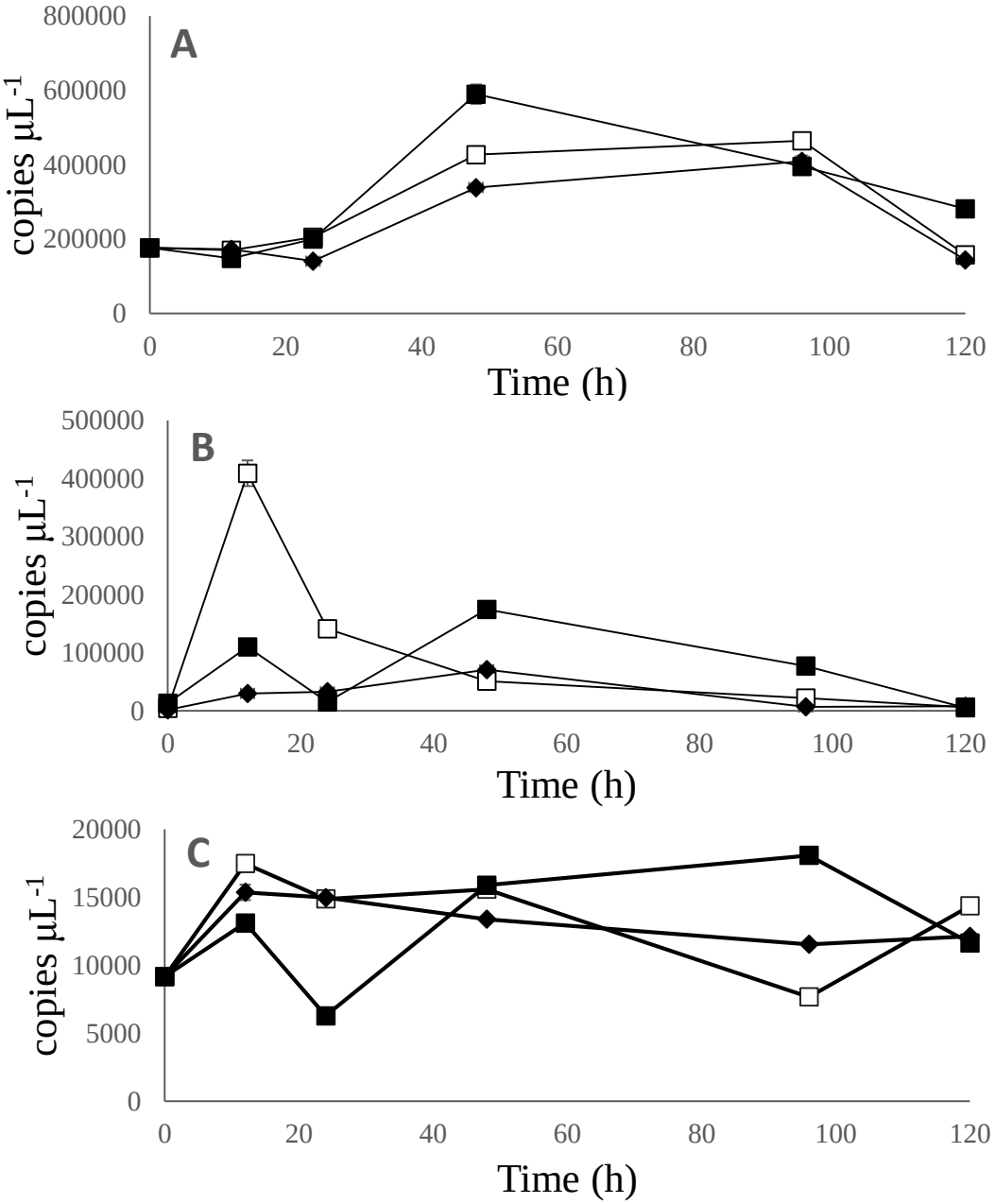

Figure 3. Quantities of total bacteria/archaea (A), total methanogens (B), and total protozoa (C) in time course cultures treated with RLE (■) showed no changes compared NA control (□). BES (◆), a known inhibitor of methanogenesis, was used as a positive control. DNA was extracted from cultures at each time point and evaluated using qPCR compared to standard curves containing DNA of known concentrations. Cultures were incubated for 120 hours at 39°C and sampling times were 0, 12, 24, 48, 96 and 120 hours. Error bars represent error from 4 independent experiments (error bars not visible are within the markers). N=5 for each treatment in each experiment.

The *mcrA* transcript numbers appeared to decrease with treatment of RLE and was lower at all time points after 0 h. (Figure 4). The ratio of *mcrA* transcripts to genes was decreased by an average of 81.6% ± 5.0% with RLE treatments compared to NA ($P=5x10^{-12}$).

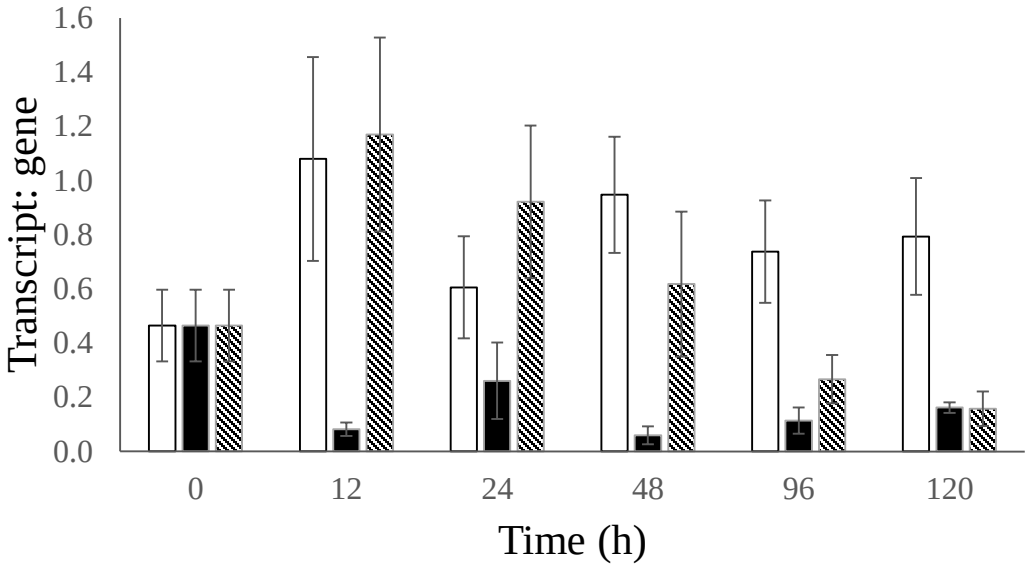

Figure 4. *mcrA* transcript:gene ratio of cultures treated with RLE (black; P values: 0.003, 0.006, 0.164, 0.007, 0.028, and 0.0208), BES (Diagonal stripes) and NA controls (white). Methanogen transcript numbers were estimated from total RNA extracted at each time point and methanogen numbers from total DNA, both using qPCR. Cultures were incubated for 120 hours at 39 °C and sampling times were 0, 12, 24, 48, 96 and 120 hours. Error bars represent standard error for 4 independent experiments, n=3 for each treatment in each experiment.

7 DISCUSSION

The current study used gas chromatography and qPCR to quantify CH_4, *mcrA* gene and transcript copy numbers, and total bacterial/archaeal and protozoal 16S rRNA and 18S rRNA gene copy numbers to evaluate the effect of RLE on *in vitro* methanogenesis in bovine rumen fluid cultures.

From this study, it is clear that RLE is able to disrupt methanogenesis in rumen fluid cultures (Figure 1, Figure 2). Interestingly, neither pure DE nor pure calcium bentonite appeared to

have any significant effect on methanogenesis except at concentrations of 75 and 100 g/L. And, while 50 and 100 g/L calcium bentonite did eliminate methanogenesis, this was attributable to the absorption of all of the water from the culture medium, an effect not observed with RLE or DE.

In time course studies, RLE supplied to cultures at 100 g/L was found to be almost as effective as BES, a reversible inhibitor of methyl-coenzyme M reductase (Lee et al., 2009). BES is often used as a positive control when studying CH_4 inhibition *in vitro* (Choi et al., 2004; Lee et al., 2009). RLE reduced CH_4 production up to 97% *in vitro*, better than many other feed additives including condensed tannins, tea saponin and sunflower oil (Guo et al., 2008; Hu et al., 2005; Huang et al., 2011; McGinn et al., 2004), nitro compounds (Anderson et al., 2010; Zhou et al., 2011) and bromochloromethane (Denman et al., 2007). RLE at a concentration of 20 g/L was also found to reduce methanogenesis by 90.5% ± 1.9% after 24 hours. In time course studies, RLE at 20 g/L was found to inhibit methane production by 59% ± 11% compared to no-additive controls after 120 hours of incubation, and significant differences were seen after 12 hours. This observation is important for future work given that RLE is currently approved for use in animal feed at this concentration. Given that treatments found to be effective *in vitro* often show significant decreases in effectiveness *in vivo* (Flachowsky and Lebzien, 2009), it is critical to carry out whole animal studies. Future work should include the development of a feed pellet or lick that incorporates a range of natural additives. This may allow for a greater impact on methanogenesis while maintaining the RLE at the approved 20 g/L level, although magnesium-mica has been fed to beef heifers at 100 g/L with no effect on dry matter intake or negative impacts on rumen function (Coffey et al., 2000).

The reduction in CH_4 produced in 20 g/L Powder RLE-treated cultures was correlated with a decrease in the *mcrA* transcript to gene ratio (Figure 4). Previous studies have quantified decreases in the presence, or transcriptional activity, of methanogens by quantifying 16S rRNA genes, or *mcrA* genes and transcripts, in correlation with a decrease in CH_4 production (Denman et al., 2007; Hook et al., 2009; Zhou et al. 2011). Given that dead cells may still be detected using qPCR of the *mcrA* gene, and that the rate of methanogenesis is controlled by cellular activity rather than strict cell numbers (Röling, 2007), monitoring *mcrA* gene to transcript copy number is advisable (Freitag and Prosser, 2009). After five days, the total number of *mcrA* gene copies was not significantly reduced compared to the control, while the number of transcripts was reduced by up to 80%, indicating that the material had a stronger impact on the methanogen activity rather than on the population size. Surprisingly, BES did not appear to have a significant effect on *mcrA* gene or transcript copy numbers, even though it was previously found to decrease methanogen numbers in a dose dependent manner (Lee et al., 2009).

In 1994, Finlay et al. discovered that ciliate protozoa are prominent single celled organisms found in the rumen of cattle which can harbour endosymbiotic methanogens providing direct transfer of H_2 for their use. Morgavi et al., (2010) published a review of the mechanisms of rumen methanogenesis, and a meta-analysis of relevant *in vivo* studies provided strong evidence that protozoa play a key role in the process. In this study, qPCR examining total bacteria, archaea and protozoa was done targeting the 16S rRNA and 18S rRNA genes. The 18S rRNA is often used as an indicator of the presence of protozoa in the rumen and has been studied by researchers examining the effects of weaning, condensed tannins, defaunation, and the use of ionophores on protozoal communities and their link to cattle rumen methanogenesis

(Karnati et al., 2003; Sylvester et al., 2003; Hegarty et al., 2008; Patra et al., 2006; Beauchemin et al., 2008; Patra, 2012). These researchers found that decreasing rumen protozoal numbers is correlated with a decrease in CH_4 production, though many of the methods used may leave chemical residues in the animal products, and do not have long lasting effects. In this study, RLE treatments were not found to have any effect on the abundance of protozoa or bacteria (Figure 3), which, considering RLE may function in a manner similar to other bentonite products, supports some research which has found no significant effect of bentonite on protozoa in ruminants (Ivan et al., 1992). Other silicates, such as magnesium-mica fed to beef heifers at up to 100 g/L feed (Coffey et al., 2000), certain dolomites (Varadyova et al., 2007) and caustic calcinated magnesites (Varadyova et al., 2006) also did not affect protozoal numbers even if methanogenesis was reduced.

Besides methanogenesis, H_2 can also be used for the production of volatile fatty acids (VFAs) such as propionate and acetate. The levels of VFAs found in the rumen can be an indication of animal feed efficiency, and decreases in non-reduced acetate to reduced propionate ratios in the rumen has been found to coincide with a decrease in CH_4 production (Christophersen et al., 2007). The effects of RLE on the VFA levels in the rumen, and the ratio of acetate: propionate should be examined to assess its feasibility of RLE as a feed additive for cattle.

Given the two lines of *in vitro* evidence presented here, Powder RLE has potential to reduce rumen methanogenesis, although *in vivo* work would be required to determine if this effect is transferable to live animals. In addition, with 2% (20 g/L) RLE currently approved as a feed additive for cattle, more detailed study of the impact of 20 g/L RLE in combination with other known inhibitors that may be best used at low doses should be carried out. A deeper understanding of the effect of RLE on rumen methanogenesis will come through metagenomic

and metatranscriptomic studies of bacterial, archaeal, fungal and protozoan communities both *in vitro* and *in vivo*, as well as studies of its effects on volatile fatty acid production (VFA) as a means to determine its overall effects on feed efficiency.

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9 APPENDIX

Table 1.Calculations used to determine CH_4 concentrations in culture headspace. CH_4 was measured directly from culture headspace and concentrations were determined by comparison with a standard curve. Atmospheric temperatures and pressures as well as culture bottle pressures were taken into account to calculate headspace volume and the total nmol of CH_4 in each bottle head space.

Value	Calculation
Headspace (HS) volume STP (mL)	$Measured\ HSvol\ (mL) * \frac{273}{273 + atm\ temp\ (℃)} * \frac{atm\ press(mmHg) + (HSpress\ (kPa) * 7.5)}{760}$
Total nmol/bottle	$\frac{nmol\ injected\ (\ calc\ from\ standard\ curve)}{volume\ injected\ (mL)} * HS\ vol\ STP(mL)$

Table 2. Primers used for qPCR analysis targeting total protozoa, total bacteria/archaea, and methanogen *mcrA* genes.

Target	Primer set	Forward Sequence	Reverse Sequence	Reference
Protozoa	316f/ 538r	GCTTCGWTGGTAGTGTTATT	CTTGCCCTCYAATCGTWCT	Sylvester et al., 2004
mcrA	qmcrA-f /qmcrA-r	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC	Denman et al., 2007
16S rRNA Universal	519-f/ 915-r	GTGCTCCCCCGCCAATTCCT	CAGCMGCCGCGGTAANWC	Jeyanathan et al., 2011

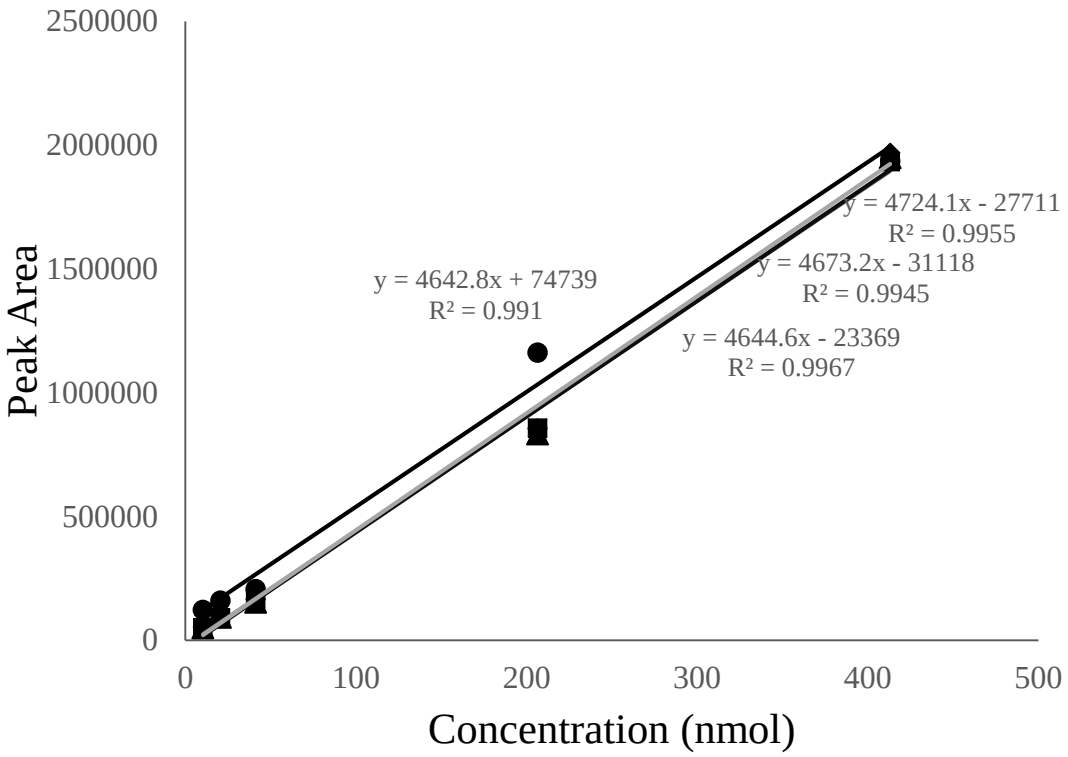

Figure 5. CH_4 standard curves from all four screening experiments were very repeatable and all had R^2 values of 0.99 or higher. Methane standards of 2.5, 5, 10, 20, 50 and 100 mL/L were prepared using CH_4 (Praxair Canada Inc., Kamloops, BC, Canada; grade 3.7, 99.97% pure) injected in different volumes into 11-mL glass vials (Labco Lmt., Lampeter, Ceredigion, UK) (Nelson, 1971). A new standard curve was prepared daily.

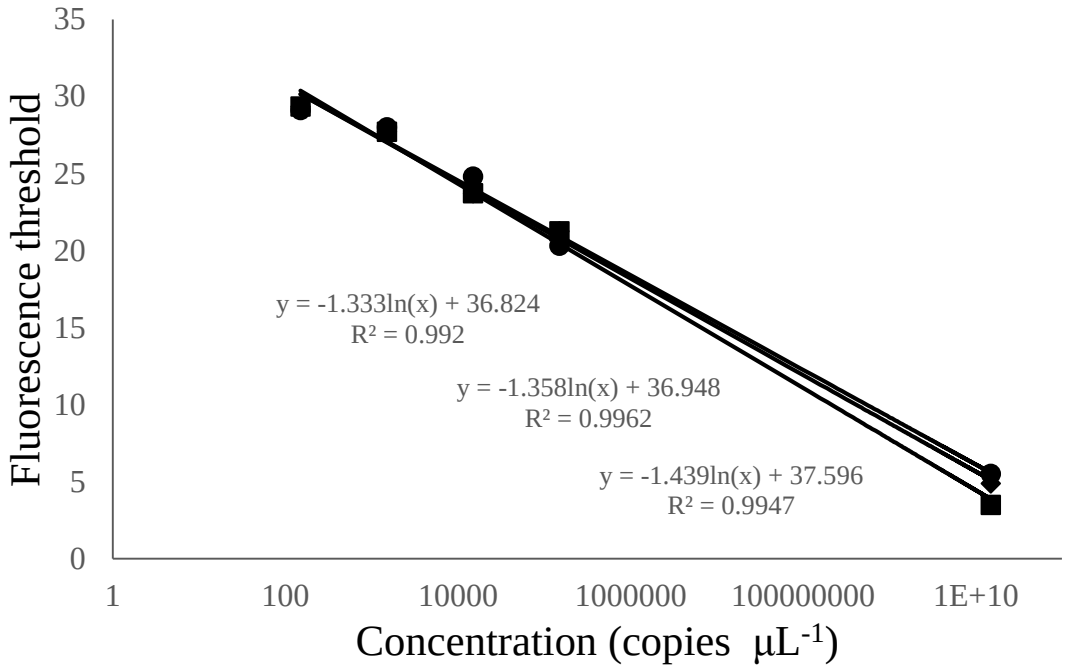

Figure 6. Total bacteria qPCR standard curves were found to be repeatable between trials. Standard curves for the quantification of total bacteria were produced using 16S rRNA gene template from *Gordonia sp*. NB4-1Y. DNA was quantified using a Qubit Fluorometer and 1:10 serial dilutions were performed to make standard curves from 2.5×10^8 copies to 250 copies per µL.