Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin® and the Hawaiian marine algae Chaetoceros on ruminal fermentation in vitro☆


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A R T I C L E   I N F O

Article history:
Received 19 March 2008
Received in revised form 1 December 2008
Accepted 1 December 2008
Available online 11 April 2009

Keywords:
Nitroethane
Lauric acid
Marine algae
Methane
Rumen

A B S T R A C T

The effects of several methane-inhibitors on rumen fermentation were compared during three 24 h consecutive batch cultures of ruminal microbes in the presence of nonlimiting amounts of hydrogen. After the initial incubation series, methane production was reduced greater than 92% from that of non-treated controls (25.8 ± 8.1 μmol ml⁻¹ incubation fluid) in cultures treated with nitroethane, sodium laurate, Lauricidin® or a finely-ground product of the marine algae, Chaetoceros (added at 1, 5, 10 and 10 mg ml⁻¹, respectively) but not in cultures treated with sodium nitrate (1 mg ml⁻¹). Methane production during two successive incubations was reduced greater than 98% from controls (22.5 ± 3.2 and 23.5 ± 7.9 μmol ml⁻¹, respectively) by all treatments. Reductions in amounts of volatile fatty acids and ammonia produced and amounts of hexose fermented, when observed, were most severe in sodium laurate-treated cultures. These results demonstrate that all tested compounds inhibited ruminal methane production in our in vitro system but their effects on fermentation differed.

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1. Introduction

Ruminal digestion of low quality feedstuffs provides the host volatile fatty acids and microbial protein to support energy requirements for sustenance, growth and work. Only about 10–35% of dietary energy consumed by the ruminant is conserved, however. Improvements in digestive efficiency could improve ruminant animal production while lowering input costs and undesired environmental impacts (Varga and Kolver, 1997). Ruminal methane production, for instance, results in the inefficient conversion of potentially energy-yielding substrates into a form that can not be conserved by the host. Methane emissions represent losses of up to 15% of gross energy intake for forage-fed cattle and losses of 2–4% for cattle consuming diets rich in readily fermentable substrates (Johnson and Johnson, 1995; Van Nevel and Demeyer, 1996). Methane is also a greenhouse gas implicated as a contributor to global warming. In the United States, approximately 21% of the total methane production is from enteric fermentation and ruminants are major contributors (EPA, 2006). Consequently, strategies are sought to reduce rumen methane production.

One strategy for reducing ruminal methane production is to provide alternative electron acceptors that more effectively consume reducing equivalents produced during fermentation so as to redirect electron flow away from the reduction of carbon dioxide to methane (Anderson and Rasmussen, 1998; Sar et al., 2004, 2005a,b). Other strategies involve supplementing ruminant diets with anti-methanogenic compounds that inhibit methanogens directly or inhibit biochemical reactions involved in the production of methane. Nitrocompounds such as nitroethane inhibit ruminal methanogenesis by as much as 90% in vitro (Anderson et al., 2003) and greater than 43% in vivo (Anderson et al., 2006) via inhibition of formate and hydrogen oxidation, reducing substrates used by ruminal methanogens (Anderson et al., 2008). Nitroethane is also known to be utilized as a terminal electron acceptor by the ruminal bacterium Dentitrobacterium detoxificans, and thus has the potential to consume reducing equivalents at the expense of methane production (Anderson et al., 2000). Additionally, the medium chain fatty acid lauric acid inhibits ruminal methanogenesis by...
as much as 89% in vitro (Dohme et al., 2001; Soliva et al., 2003) and by up to 76% in vivo (Machmüller et al., 2002), and the long chain fatty acid hexadecatrienoic acid from the marine algae Chaetoceros inhibited in vitro methane production by 97% (Ungerfeld et al., 2005). Medium and long chain fatty acids are thought to inhibit the growth of Gram-positive and methanogenic bacteria via absorption and disruption of cell membranes (Galbraith and Miller, 1973; Soliva et al., 2003). A direct comparison of methane accumulation in the presence of these different compounds is not available. Accordingly, the primary objective of the present study was to compare the effects of nitrate, nitroethane and lauric acid (as the sodium salts), and the marine algae Chaetoceros on ruminal methane production in vitro. Lauricidin\(^\text{a}\) is a glycerol monoester of lauric acid which exhibits bactericidal activity against Gram-positive bacteria similar to that of lauric acid (Kabara et al., 1972). Consequently, Lauricidin\(^\text{a}\) was included as another treatment to evaluate the methane-inhibiting potential of this monoester.

### 2. Methods

Tests for effects of inhibitors on ruminal methane production were accomplished by consecutive batch culture (Theodorou et al., 1987) of mixed populations of ruminal microbes in 18 \ times 15 cm crimp top culture tubes containing 0.2 g ground alfalfa, 9 ml rumen fluid based medium and test compounds as indicated. Alfalfa was included as a forage substrate to simulate daily intake of forage components. The rumen fluid based medium contained 40% clarified rumen fluid and salts as in Medium B (Anderson and Rasmussen, 1998). Nitroethane and sodium nitrate were supplied to sterilized media via addition of 50 \(\mu\)l or 100 \(\mu\)l of 200 mg or 100 mg ml\(^{-1}\) filter-sterilized stock solutions, respectively. The sodium salt of nitroethane was prepared as described by Majak et al. (1986) and diluted appropriately with deionized water. Sodium laurate, Lauricidin\(^\text{a}\) and a ground product of the marine algae were added as dry additions to each tube before addition of medium. Sodium nitrate, nitroethane and sodium laurate were purchased from Sigma–Aldrich (St. Louis, MO, USA); Lauricidin\(^\text{a}\) was graciously provided by Dr. Jon Kabara (Bradenton, FL, USA). The marine algae Chaetoceros was produced and harvested from an open continuous microalgae culture system at the Anuenue Fisheries Center, Sand Island, Oahu, Hawaii, and provided as a ground preparation (approximately 1 mm particle size) containing 2.46 mg hexadecatrienoic acid g\(^{-1}\) of algae dry weight. Freshly collected ruminal fluid obtained from a cannulated Holstein–Friesian cow grazing rye grass pasture was used as inoculum (1 ml) for each tube within the initial incubation series. A consecutive batch culture technique was used to allow opportunity to observe potential adaptations that were anticipated, particularly with nitrate-treated populations. After an initial 24 h incubation (39 °C), 1 ml volume from each culture was transferred to a new series of tubes containing fresh medium without (controls) or with respective test compounds and incubated as above for 24 h. The process was repeated for the third incubation series. Hydrogen gas was provided in excess via use of a hydrogen:carbon dioxide (50:50) gas phase to minimize potential confounding effects of the different inhibitors on amounts of hydrogen produced from digestion of media components. Controls and treatments were incubated in triplicate during each series. Because populations within each tube had an opportunity to respond independently upon each successive transfer, each was considered an independent experimental unit. Gas composition in headspace gas of each culture sample after each 24 h incubation series was determined by gas chromatography (Allison et al., 1992); gas volume was measured via insertion of a 30 cc lubricated air-tight glass syringe fitted with an 18 gauge needle through the stopper of each tube and volume displacement was recorded. Fluid samples collected after 24 h incubation for were analyzed colorimetrically for determination of ammonia (Chaney and Marbach, 1962); lactate was measured enzymatically (Hohorst, 1965) and volatile fatty acids were measured by gas chromatography (Hinton et al., 1990). Values reported are net amounts produced and were calculated as the difference between concentrations measured in fluid samples collected after each 24 h incubation minus initial concentrations. Amounts of hexose fermented were calculated as the sum of \(\frac{1}{2}\) acetate + \(\frac{1}{2}\) propionate + butyrate + valerate + \(\frac{1}{2}\) lactate (DeMeyer, 1991). Hydrogen balance was determined using the equations described by Ungerfeld et al. (2003) except modified to include a term for reducing equivalents (equiv.) generated from the oxidation of supplied \(\text{H}_2\) which was calculated as the difference between hydrogen supplied and residual hydrogen measured at the end of each incubation. Additionally, the term for \(\text{H}_2\) produced from fermentation was omitted because headspace \(\text{H}_2\) concentrations (ranging from 31 to greater than 3 \(\mu\)mol ml\(^{-1}\)) exceeded concentrations reported to inhibit the ability of ruminal microbes to dispose of reducing equivalents generated during glycolysis via hydrogenase catalyzed production of hydrogen (Miller, 1995; Van Nevel and Demeyer, 1996). Tests for main effect of treatment, incubation series and their interactions were conducted using a general analysis of variance with a Turkey’s multiple comparison of means (STATISTIX\(^\text{R}\) Analytical Software, Tallahassee, FL, USA).

### 3. Results

#### 3.1. Comparative effects of inhibitors on total gas and methane production and on residual hydrogen concentrations

A main effect of treatment was observed on total gas produced during the consecutive batch cultures, with 47% and 75% less total gas produced by Lauricidin\(^\text{a}\) and laurate-treated cultures, respectively, than that produced by controls (Table 1). Amounts of gas produced by cultures treated with the marine algae, nitrate and nitroethane did not differ from controls (Table 1). A main effect of incubation series or a treatment by incubation series interaction on total gas production was not observed (\(P > 0.05\)). A main effect of treatment by the respective methane inhibitors on methane production was observed (Table 1) as well as a main effect of incubation series and a treatment by incubation series interaction (Fig. 1). All inhibitors except nitrate caused reduction in amounts of methane produced at the end of the initial incubation series when compared to controls containing no added inhibitor. Methane production in nitrate-treated cultures was reduced after the second and third incubation series with amounts produced being comparable to those observed in incubations treated the other inhibitors (Fig. 1). Differences in hydrogen recovery due to treatment, incubation series or their interaction were not observed (\(P > 0.05\); Table 1).

#### 3.2. Effects on lactate, volatile fatty acid and ammonia production

Small amounts of lactate accumulated within all cultures but these were not affected by treatment (Table 1), incubation series or their interaction (\(P > 0.05\)). A main effect of treatment was observed on the production of all individual volatile fatty acids measured as well as on total volatile fatty acid production (Table 1), but main effects of incubation series and treatment by incubation series interactions were only observed for propionate, butyrate and valerate (Fig. 2A–C). Main effect treatment means for acetate production were higher for control and nitrate-treated cultures, were intermediate for marine algae, nitroethane and laurate-treated
Table 1
Main effects of treatment on fermentation characteristics during 3 consecutive batch cultures of ruminal microbes with or without addition of select methane inhibitors.

<table>
<thead>
<tr>
<th>Fermentation characteristic</th>
<th>Treatmenta</th>
<th>P value</th>
<th>SEMb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Sodium nitrate</td>
<td>Sodium laurate</td>
</tr>
<tr>
<td>Headspace measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total gas produced (ml)</td>
<td>7.2e</td>
<td>5.8f</td>
<td>4.9f</td>
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<tr>
<td>Methane produced (µmol ml⁻¹)</td>
<td>21.24e</td>
<td>7.92d</td>
<td>0.22g</td>
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<tr>
<td>Residual hydrogen (µmol ml⁻¹)</td>
<td>3.40</td>
<td>3.48</td>
<td>3.57</td>
</tr>
<tr>
<td>Culture fluid measurements</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (µmol ml⁻¹)</td>
<td>62.77e</td>
<td>71.40e</td>
<td>40.75f</td>
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<tr>
<td>Propionate (µmol ml⁻¹)</td>
<td>34.42f</td>
<td>30.72e</td>
<td>23.88a</td>
</tr>
<tr>
<td>Butyrate (µmol ml⁻¹)</td>
<td>10.63f,1</td>
<td>7.55d</td>
<td>11.27a</td>
</tr>
<tr>
<td>Valerate (µmol ml⁻¹)</td>
<td>2.54f,4</td>
<td>1.53b,1</td>
<td>3.13f</td>
</tr>
<tr>
<td>Isobutyrate (µmol ml⁻¹)</td>
<td>1.17f,1</td>
<td>1.17a</td>
<td>1.17d</td>
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<tr>
<td>Isovalerate (µmol ml⁻¹)</td>
<td>0.70f</td>
<td>0.68b</td>
<td>0.71i</td>
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<td>Total volatile fatty acids (µmol ml⁻¹)</td>
<td>112.24f</td>
<td>113.06e</td>
<td>81.10a</td>
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<td>Acetate:Propionate ratio</td>
<td>1.81f</td>
<td>2.41i</td>
<td>1.82f</td>
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<td>Lactate (µmol ml⁻¹)</td>
<td>0.17</td>
<td>0.16</td>
<td>0.21</td>
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<tr>
<td>Ammonia (µmol ml⁻¹)</td>
<td>5.57f</td>
<td>8.17j</td>
<td>5.36l</td>
</tr>
<tr>
<td>Stoichiometric calculations</td>
<td></td>
<td></td>
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<tr>
<td>Hexose fermented (µmol ml⁻¹)</td>
<td>61.85e</td>
<td>60.22e</td>
<td>47.07f</td>
</tr>
<tr>
<td>Reducing equiv. generated (µmol H₂ equiv. ml⁻¹)</td>
<td>243.47f</td>
<td>244.16e</td>
<td>193.41f</td>
</tr>
<tr>
<td>Reducing equiv. consumed (µmol H₂ equiv. ml⁻¹)</td>
<td>185.40f</td>
<td>114.52d</td>
<td>84.66g</td>
</tr>
</tbody>
</table>

a Treatments (per ml of incubation fluid) were controls, no addition; 1 mg sodium nitrate, 1 mg nitroethane; 5 mg sodium laurate; 5 mg Lauricidin® or 10 mg marine algae.
b Values are means and SEM from a general analysis of variance test for main effect of treatment.

c Amounts of hexose fermented calculated as 1/2 acetate + 1/2 propionate + butyrate + valerate + 1/2 lactate (DeMeyer, 1991). Reducing equivalents (equiv.) generated calculated as 2 equiv. acetate + 1 equiv. propionate + 4 equiv. butyrate + 3 equiv. valerate + 1 equiv. lactate + 1 equiv. NE; H₂ equiv. oxidized; Hz equiv. oxidized calculated as the difference between hydrogen supplied and residual hydrogen. Reducing equiv. consumed calculated as 2 equiv. propionate + 2 equiv. butyrate + 4 equiv. valerate + 1 equiv. lactate + 4 equiv. CH₄ (Ungerfeld et al., 2003).
d–i Means within rows with unlike superscripts differ (P < 0.05).

Fig. 1. Treatment by incubation series interaction observed on the production of methane (P < 0.0001) during three consecutive batch cultures of ruminal microbes without (controls) or with addition of 1 mg sodium nitrate, 1 mg nitroethane; 5 mg sodium laurate; 5 mg Lauricidin® and 10 mg marine algae per ml incubation fluid. Values are least-squares means from n = 3 cultures during each incubation series and bars with unlike letter designations differ (P < 0.05).

Figures, captions, and tables are integrated into the main text, and the text flows naturally as if reading it naturally.
and nitrate-treated cultures was lower than that by controls or the marine algae-treated cultures but the differences were not necessarily significant (Fig. 2B). Main effect treatment means for valerate were highest for nitroethane and the marine algae-treated
cultures, lowest in nitrate and laurate-treated cultures and intermediate in control and Lauricidin®-treated cultures. Comparison of least-squares means revealed that valerate production increased during successive incubation for all cultures except those treated with laurate or nitrate, but again the increase was not necessarily significant (Fig. 2C).

In the case of isobutyrate, amounts produced by the marine algae-treated cultures did not differ from amounts produced by nitroethane-treated cultures but were higher than amounts produced by controls and the other treated cultures (Table 1). Amounts of isobutyrate produced by laurate-treated cultures did not differ from those produced by nitrate-treated cultures but were lower than controls and all the other treatments (Table 1). Amounts of isovalerate produced were highest in the marine algae-treated cultures but did not differ between controls and the other treated cultures (Table 1).

Total volatile fatty acid production was decreased (P < 0.05) 45%, 54% and 28% from that of controls in Lauricidin, laurate and nitroethane-treated cultures, respectively, but not in nitrate or marine algae treated cultures (Table 1).

A main effect of treatment was observed on ammonia accumulations (Table 1) as were effects of incubation series and a treatment by incubation series interaction (Fig. 3). Main effect mean ammonia accumulations were highest (P < 0.05) for the marine algae-treated cultures, second highest (P < 0.05) for the nitrate-treated cultures and lowest (P < 0.05) for laurate and Lauricidin®-treated cultures. Ammonia accumulations for control and nitroethane-treated cultures were intermediate (Table 1). Comparison of least-squares means revealed that ammonia accumulations were reduced after the second and third incubation series compared to the first incubation series in Lauricidin®-treated cultures but did not differ between incubation series in controls or any of the other treated cultures (Fig. 3).

### 3.3. Effects on estimates of hexose fermented and hydrogen balance

A main effect of treatment observed on estimated amounts of hexose fermented (Table 1) but a main effect of incubation series and a treatment by incubation series interaction were not observed (P > 0.05). Laurate and Lauricidin®-treated cultures fermented 46.8% and 55.6% less hexose than controls (Table 1). Estimates of amounts of hexose fermented by nitrate, marine algae and nitroethane-treated cultures did not differ from controls (Table 1).

A main effect of treatment was observed on amounts of reducing equivalents generated during fermentation (Table 1) with fewer reducing equivalents generated by laurate and Lauricidin®-treated cultures than control, nitrate, the marine algae or nitroethane-treated cultures. A main effect of incubation series or a treatment by incubation series interaction were not observed (P > 0.05). A main effect of treatment was observed on amounts of reducing equivalents incorporated into fermentation products, with main effect means being highest for controls and lowest for laurate-treated cultures, with means for the other treatments being intermediate (Table 1). A main effect of incubation series and a treatment by incubation series interaction were also observed (Fig. 4). A comparison of least-squares means revealed that estimated amounts of reducing equivalents incorporated into fermentation products were reduced after the second and third incubation series compared to the first incubation series in the nitrate-treated cultures but did not differ between incubation series in controls or any of the other treated cultures (Fig. 4).

### 4. Discussion

With the exception of nitrate, all inhibitors caused greater than 92% reduction (P < 0.05) in amounts of methane produced during the initial incubation series. There is some evidence that a potential lag in methane-reducing activity occurred in the cultures treated with Lauricidin® and the marine algae, as methane production was noticeably, but not significantly, higher in these incubations than those treated with nitroethane and laurate (Fig. 1). Lauricidin®, a glycerol monoester of lauric acid, and it is reasonable to suspect that hydrolysis of the glycerol moiety was required before inhibitory activity would be observed. Likewise, the long chain fatty acid from the ground marine algae product, hexadecatrienoic acid, may also not have been immediately available within the incubations. In subsequent series, methane production was inhibited greater than 96% in all incubations containing the inhibitors, including those treated with nitrate, when compared to controls (P < 0.05). Differences in residual hydrogen concentrations measured at the end of the 24 h incubation were not observed between controls or any of the treatments. This is not unexpected as hydrogen was provided in excess to promote comparisons between the effects of the different inhibitors on methanogenesis when hydrogen, as a reducing substrate, was nonlimiting. Consequently, hydrogen concentrations were always high, greater than

![Fig. 3. Treatment by incubation series interaction observed on the accumulation of ammonia (P = 0.0002) during three consecutive batch cultures of ruminal microbes without (controls) or with addition of 1 mg sodium nitrate, 1 mg nitroethane; 5 mg sodium laurate; 5 mg Lauricidin® and 10 mg marine algae per ml incubation fluid. Values are least-squares means from n = 3 cultures during each incubation series and bars with unlike letter designations differ (P < 0.05; SEM = 0.48).](image-url)
At these hydrogen concentrations, it is likely that reducing equivalents produced during fermentation were redirected away from hydrogen production in all the cultures, even the controls, as hydrogen concentrations of 1 kPa (equivalent to nearly equal concentrations within all the incubations, which further indicates that disposal of excess reducing equivalents via hydrogen production was inhibited as lactate usually does not accumulate to appreciable concentrations in the rumen.

The lack of a methane-reducing effect of the nitrate treatment at the end of the first incubation series (Fig. 1) is not unexpected as nitrate is not a potent inhibitor of methanogenesis per se. However, the consecutive batch culture technique likely allowed for an adaptation of the microbial population to the nitrate treatment as evidenced by methane production being reduced in nitrate-treated cultures comparably to that observed with the other inhibitors after the second and third incubation series (Fig. 1). Ruminal adaptation to high nitrate diets has been demonstrated and is a consequence both of a rapid induction of nitrate-reducing activity as well as an enrichment of nitrate-respiring populations (Allison and Reddy, 1984). It is known that high ruminal nitrate reductase activity, whether achieved via adaptation of the rumen microflora or via inoculation with specialized nitrate and nitrite-reducing microbes, can effectively compete against methanogenesis for available reductant (Allison and Reddy, 1984; Anderson and Rasmussen, 1998; Sar et al., 2004, 2005a,b). However, reductant was provided in excess in the present experiment and thus would not be considered to be limiting for methanogenesis. Thus in the present experiment it is likely that a transient accumulation of nitrite, produced as an intermediate during the reduction of nitrate, may have contributed to the reduction of methane in the nitrate-treated cultures. Nitrite is known to be toxic to some methanogens (Klüber and Conrad, 1998) and to inhibit rumen methanogenesis (Sar et al., 2005b). The potential ruminal accumulation of nitrite and its risk of causing methemoglobinemia in livestock has long been a deterrent to the use of nitrate as a feed additive to reduce ruminal methanogenesis. Recent work, however, suggests that the risk of ruminal nitrite accumulation may be mitigated by the co-supplementation of nitrate with prebiotic substrates or nitrite-reducing bacteria resulting in enhanced rates in the conversion, and thus detoxification, of nitrite to ammonia (Anderson and Rasmussen, 1998; Sar et al., 2004, 2005a,b).

An attractive aspect of using electron acceptors such as nitrate as alternative electron sinks is that fermentative efficiencies associated with microbial interspecies hydrogen transfer reactions are conserved, as illustrated by nearly identical volatile fatty acid production profiles observed in control and nitrate-treated cultures (Table 1). For instance, except for a 39.8% decrease in the amount of valerate produced, volatile fatty acid production by nitrate-treated cultures did not differ from controls and consequently, estimates of amounts of reducing equivalents generated did not differ (Table 1). In continuous flow culture, treatment of ruminal populations with 5 or 10 μmol nitrate ml⁻¹ incubation fluid decreased production of acetate, propionate and butyrate, but co-addition of 10 μmol nitrate or 2 μmol nitrite ml⁻¹ with an E. coli construct possessing enhanced nitrite reductase activity increased rates of nitrite detoxification and production of these fatty acids (Sar et al., 2005b). Similarly, administration of 1.3 g sodium nitrate per kg.⁷⁵ body weight to sheep resulted in decreased ruminal accumulation of acetate, propionate and butyrate. In this case co-administration of nitrate with the nitrite reductase-enhanced E. coli construct increased acetate but decreased propionate and butyrate production (Sar et al., 2004, 2005a). In the present experiment, the accumulation of ammonia was higher in the nitrate-treated cultures than in controls (Table 1) but was likely due to the dissimilatory reduction of the added nitrate to ammonia by nitrate-reducing microbes. This observation is supported by the finding that production of the branched chain volatile fatty acids, products of amino acid fermentation (Allison, 1978), by the nitrate-treated cultures did not differ from controls (Table 1). Amounts of reducing equivalents consumed by the nitrate-treated cultures were lower than those consumed by controls (Table 1) but because nitrate was not measured in the present study, this estimate does not include the likely consumption of 48 μmol H₂ equivalents ml⁻¹ for the reduction of approximately 12 μmol added nitrate ml⁻¹ to ammonia. Assuming complete reduction of nitrate and nitrite by the microbial populations by 24 h of incubation, as observed during continuous flow culture of non-adapted ruminal cultures.
microbes with 10 μmol ml⁻¹ added nitrate (Sar et al., 2005b), then 161 μmol H₂ equivalents ml⁻¹ were consumed by the nitrate-treated cultures in this experiment which does not differ (P > 0.05) from that of controls.

Evidence indicates that nitroethane may also serve as an alternative electron sink, as greater than 88% of the added nitroethane was metabolized and numbers of nitro-reducing bacteria were enriched greater than 1000-fold numbers during consecutive batch culture ruminal microbes with additions of 4.5 and 9.0 μmol nitroethane ml⁻¹ (Gutierrez-Bañuelos et al., 2008). Evidence for enrichment was also obtained in an in vivo study where rates of ruminal nitroethane-metabolism increased in steers administered 80 or 160 mg nitroethane 80 mg kg⁻¹ body weight d⁻¹ compared to non-treated controls (Gutierrez-Bañuelos et al., 2007). Currently, the Gram-positive, obligate respiratory anaerobe, D. detoxificans, is the only known ruminal bacterium to possess appreciable nitroethane-metabolizing ability (Anderson et al., 2000). While normally present at less than 10⁶ organisms ml⁻¹ in the rumen of ruminants having no known exposure to nitroethane or related nitrocompounds, D. detoxificans was enriched to greater than 10⁶ of organisms ml⁻¹ via consecutive batch culture in medium supplemented with 3-nitro-1-propanol (Anderson et al., 1996). This bacterium conserves energy for growth via the oxidation of hydrogen, formate or to a lesser extent lactate for the reduction of suitable electron acceptors such as nitrate, nitroethane, 2-nitro-1-propanol, 2-nitroethanol and the phytoxins, 3-nitro-1-propanionic acid and 3-nitro-1-propanol (Anderson et al., 2000). In the present study, the estimated amount of reducing equivalents consumed within nitroethane-treated cultures was decreased compared to controls. However, because nitroethane concentrations were not measured in the present study this estimate does not include the potential consumption reducing equivalents for the reduction nitroethane to ethanamine which presumably consumes 3 mol hydrogen per mol nitroethane similar to that reported for the reduction of nitro-ethanol to aminoethanol (Angermäier and Simon, 1983). If 88% of the 13.3 μmol ml⁻¹ added nitroethane were reduced, as was observed in a similarly conducted experiment (Gutierrez-Bañuelos et al., 2008), then the consumption of approximately 35 μmol H₂ equivalents would account for 42% of the 84 μmol H₂ equivalents spared due to the decreased amounts (21 μmol ml⁻¹) of methanone produced (Table 1).

For cultures treated with nitroethane, amounts of total volatile fatty acids produced were decreased compared to controls, due to decreased production of acetate and propionate (Table 1). Consequently, estimates of amounts of reducing equivalents generated and amounts of hexose fermented by the nitroethane-treated cultures were 20.6% and 23.9% lower, respectively, than in controls but these differences were not significant (Table 1). A comparison of least-squares means revealed that less propionate and more valerate were produced by the nitroethane-treated cultures during the second and third incubation series than during the first series (Fig. 1). These results conflict with those from a similarly conducted study where acetate and propionate concentrations increased during the first three consecutive batch cultures of ruminal microbes treated with 4.5 or 9 μmol ml⁻¹ nitroethane and did not differ from untreated controls after 16 consecutive incubations (Gutierrez-Bañuelos et al., 2008). It is possible that the nitroethane dose in the present study, equivalent to 13 μmol ml⁻¹, may have been high enough to be toxic to the fermentative bacterial population. In support of this later hypothesis, nitroethane administered to ewes and steers to achieve 4.5 and 7.2 μmol nitroethane ml⁻¹ ruminal fluid had no effect on volatile fatty acid production whereas a dose estimated to achieve 14.3 μmol nitroethane ml⁻¹ reduced volatile fatty acid concentrations compared to non-treated controls (Anderson et al., 2006; Gutierrez-Bañuelos et al., 2007). However, treatment with a level similar to the present study (12 μmol nitroethane ml⁻¹) had no effect on volatile fatty acid production during 24 h batch culture of avian cecal microbes incubated under an 80:20 H₂:CO₂ gas phase (Saengkerdsuub et al., 2006) which suggests that undetermined differences may have existed between the populations studied in the two different experiments. Results from even earlier experiments provide no further clarity with respect to these two hypotheses as treatment with 12 μmol nitroethane ml⁻¹ decreased the production of acetate and propionate during one 24 h batch culture of ruminal microbes incubated under 100% CO₂ but increased production of these volatile fatty acids in another experiment conducted similarly (Anderson et al., 2003). Nitroethane has been reported to cause greater than 42% inhibition of formate oxidation and greater than 16% inhibition of hydrogen oxidation within mixed populations of ruminal bacteria (Anderson et al., 2008) and it is possible that formate, which was not measured here, may have accumulated as an atypical ruminal electron sink product. It has been reported that some ruminal microbes can reduce CO₂ or shift fermentation to produce formate reducing equivalents are in excess (Asanuma et al., 1999; Miller and Wolin, 1973; Shi et al., 1997). As observed in earlier studies, ammonia accumulations did not differ between nitroethane treated cultures and controls (Anderson et al., 2003, 2008; Gutierrez-Bañuelos et al., 2008).

In the present incubations, stoichiometric estimates of reducing equivalents produced from fermentation, expressed as μmol H₂ equivalents ml⁻¹ incubation fluid, were reduced from that of controls in cultures treated with Lauricidin® or laurate (Table 1). Total gas production was also reduced (P < 0.05) from that produced by control cultures in cultures treated with Lauricidin® or laurate (Table 1). When considered with the decrease in methane production, the decrease in total gas production observed due to Lauricidin® or laurate indicates that fermentation was inhibited in these incubations. The observed decreases in volatile fatty acid production and stoichiometric estimates of hexose fermented in the Lauricidin®- and laurate-treated cultures further indicate that these treatments inhibited fermentation. In contrast to the laurate-treated cultures, propionate production was not reduced in the Lauricidin®-treated cultures which is likely due to the hydrolysis and subsequent fermentation of approximately 18 μmol glycerol ml⁻¹ to propionate. At lower treatment doses, approximately 0.6–0.7 mg lauric acid ml⁻¹ incubation fluid, and under a nitrogen gas phase, lauric acid inhibited ruminal fiber digestion during in vitro incubation of ruminal microbes, but volatile fatty acid production was not affected (Dohme et al., 2001; Soliva et al., 2004). The inhibition in fiber digestion was transient, however, with inhibition diminishing after 10 d of incubation. Conversely, Yabuuchi et al. (2006) observed decreased production of acetate and increased production of propionate during in vitro incubation of ruminal microbes with 0.3–1.2 mg lauric acid ml⁻¹ incubation fluid. Butyrate production was increased in incubations treated with 0.6 mg lauric acid ml⁻¹. At treatment levels near those administered in the present study, Hristov et al. (2004b) also observed decreased production of measured volatile fatty acids during in vitro incubation of ruminal microbes with 2.5, 5.0 and 10.0 mg sodium laurate ml⁻¹ incubation fluid, although the decreases were not as great as those observed here. Direct comparisons between these different studies is cautioned, however, as the use of a 50:50 H₂:CO₂ rather than the 100% CO₂ (Hristov et al., 2004b; Yabuuchi et al., 2006) or 100% N₂ gas phases used earlier (Dohme et al., 2001; Soliva et al., 2004) likely contributed to the more severe effect of lauric acid on volatile fatty acid production observed here. As observed by Hristov et al. (2004b), ammonia accumulation was reduced compared to controls in the laurate-treated cultures studied here (Table 1). Ammonia production was reduced in the Lauricidin®-treated cultures as well (Table 1) and this suggests an inhibition of amino acid fermentation. Aside from their membrane disrupting properties,
medium chain fatty acids and mono-esters have been shown to inhibit amino acid uptake by bacteria (Kato and Shibañakî, 1976; Shibañakî and Kato, 1978), although a direct inhibition of hyper ammonia-producing ruminal bacteria such as Clostridium amino-philum, C. sticklandii or Peptostreptococcus anaerobius (Paster et al., 1993), can not be ruled out. In in vivo studies, Hristov et al. (2004a) found no adverse effect of administering 240 g sodium laurate d−1 to dairy cattle, equivalent to approximately 3 mg ml−1 rumen contents (based on an assumed 80 l rumen volume), on ruminal volatile fatty acid or ammonia concentrations. Conversely, Machmüller et al. (2003) reported ruminal concentrations of butyrate and isovalerate were reduced in lambs fed diets supplemented with coconut oil (containing lauric acid) compared to control lambs fed non-fat supplemented diets.

Hexadecanatrienic acid has also been reported to inhibit organic matter digestibility when supplemented to in vitro rumen incubations at concentrations of up to 0.45 mg ml−1 (Ungerfeld et al., 2005). In the present experiment, addition of the marine algae product provided approximately 0.02 mg hexadecanatrienic acid ml−1 which may have been low enough to avoid negative effects on digestibility. Moreover, supplementation of 10 mg marine algae ml−1 to the incubations likely provided sufficient additional organic matter to support increased substrate fermentation, particularly of amino acids, as the production of branched chain volatile fatty acids and accumulations of ammonia, end products of amino acid fermentation, were highest in the marine algae-treated cultures (Table 1). The production of acetate, but not of propionate or butyrate, was reduced in the marine algae-treated cultures compared to controls (Table 1). Ungerfeld et al. (2005) reported decreased production of acetate, isovalerate and ammonia and increased production of propionate and valerate during in vitro incubations of ruminal microbes treated with 0.45 mg hexadecanatrienic acid ml−1 (as the free fatty acid). Hydrogen balance estimates revealed that amounts of reducing equivalents generated in the marine algae-treated cultures did not differ from the controls but because less methane was produced in the marine algae-treated cultures the amounts of reducing equivalents consumed were reduced (Table 1).

5. Conclusion

The primary objective of the present study was to compare the methane-inhibiting potential of sodium nitrate, nitroethane, sodium laurate, Lauricidin® and the marine algae Chaetoceros on ruminal methane production. Our results confirm that all of tested inhibitors reduced ruminal methane production and demonstrate that except for a potential lag for the inhibitors Lauricidin®, the inhibitors reduced ruminal methane production and demonstrate ruminal methane production. Our results confirm that all of tested inhibitors reduced ruminal methane production and demonstrate ruminal methane production.

References


