Bioresource Technology 100 (2009) 4017–4025

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

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

A.K. Božic [a], R.C. Anderson [b,*], G.E. Carstens [c], S.C. Ricke [d], T.R. Callaway [b], M.T. Yokoyama [e], J.K. Wang [f], D.J. Nisbet [b]

[a] Department of Animal Science, Faculty of Agriculture, University of Novi Sad, Novi Sad, Republic of Serbia
[b] United States Department of Agriculture/Agricultural Research Service, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit, 2881 F&B RD College Station, TX 77845, USA
[c] Department of Animal Science, Texas A&M University, College Station, Texas, USA
[d] Center for Food Safety, University of Arkansas, Fayetteville, Arkansas, USA
[e] Department of Animal Science, Michigan State University, East Lansing, Michigan, USA
[f] Department of Molecular and Biosystems Engineering, University of Hawaii, Monoa, Hawaii, USA

ARTICLE INFO

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

ABSTRACT

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, 5 and 10 mg ml^{-1}, respectively) but not in cultures treated with sodium nitrate (1 mg $m1^{-1}$). 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.

Published by Elsevier Ltd.

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 substates (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

☆ Mandatory disclaimer: Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

* Corresponding author. Tel.: +1 979 260 9317; fax: +1 979 260 9332.
E-mail address: Robin.Anderson@ars.usda.gov (R.C. Anderson).

0960-8524/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.biortech.2008.12.061

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® is a glycerol monoester of lauric acid which exhibits bactericidal activity against Grampostive bacteria similar to that of lauric acid (Kabara et al., 1972). Consequently, Lauricidin® 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 × 150 mm 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 μl or 100 μl 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® 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® 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 sampled 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 ½ acetate + ½ propionate + butyrate + valerate + ½ 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 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 H_2 produced from fermentation was omitted because headspace H_2 concentrations (ranging from 31 to greater than 3 μmol 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®8 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® 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