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Effective reduction of enteric methane production by a combination of nitrate and saponin without adverse effect on feed degradability, fermentation, or bacterial and archaeal communities of the rumen

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HIGHLIGHTS

- Addition of saponin and nitrate together markedly inhibited methane production.
- This combination increased feed degradability and total volatile fatty acids.
- Saponin–nitrate combination reduced abundances of protozoa and methanogens.
- Saponin alone and in combination with nitrate increased cellulolytic bacteria.
- Saponin plus nitrate additively lower methane with no adverse effect on digestion.

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ABSTRACT

This study evaluated the effects of Quillaja saponin (0.6 and 1.2 g/L), propynoate (4 and 8 mM), and nitrate (5 and 10 mM), alone or in combinations, on methanogenesis, fermentation, bacterial and archaeal communities, and abundances of select ruminal microbial populations. All treatment decreased methane production, but combination of all three inhibitors at high dose achieved the greatest inhibition (85%). Propynoate, alone or in combination with nitrate or saponin, decreased feed degradability and total volatile fatty acid (TVFA) concentrations. However, saponin and nitrate alone at high dose and in combination at low dose inhibited methanogenesis substantially while increasing feed degradability and TVFA concentrations. The abundances of methanogens were lowered by all inhibitors except saponin alone. *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were increased by saponin, both alone and in combination with nitrate, but inhibited by propynoate. Combination of saponin and nitrate may have practical application in mitigating methane emission from ruminants.

1. Introduction

The livestock production systems in both developing and developed economies have been facing a number of growing environmental challenges including emissions of greenhouse gases into the atmosphere and excretion of nitrogen polluting ground water (Steinfeld et al., 2006). As livestock populations continue to grow to meet the escalating demands for meat and milk products by the increasing human population, these environmental problems would be worsened unless practical and cost-effective mitigation strategies are developed and implemented. Greenhouse gas emissions from livestock are a major environmental concern because they contribute substantially, about 12–18% in CO_2-equivalent, to total global greenhouse gas emissions (Steinfeld et al., 2006; Westhoek et al., 2011). One major particular concern is over methane emission resulted from rumen fermentation of feeds, which was estimated to contribute about 37% of the total anthropogenic methane emission (Steinfeld et al., 2006). Besides, methane production in the rumen also causes a significant feed energy loss depending upon the diets. Therefore, effective mitigation of methane emission from ruminant animals, mostly through dietary interventions, has been 'holy grail' in alleviating the environmental concern caused by the livestock industry.

Several methane inhibitors have been repeatedly evaluated, primarily individually, for their efficacy to inhibit enteric methane production in ruminants (Patra, 2012). In most studies, however, the dilemma is that these inhibitors often exert adverse effects on feed intake, digestion, and rumen fermentation when added

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at concentrations high enough to achieve substantial or desirable reduction in methane production, while they result in little inhibition to methane production when added at concentration that do not reduce animal productivity or feed digestion (Patra, 2012). Nitrate, saponin, and propynoic acid are such methanogenic inhibitors commonly evaluated (van Zijderveld et al., 2010; Zhou et al., 2011, 2012; Patra and Yu, 2012). Quillaja saponin may inhibit the growth of rumen protozoa (Patra et al., 2012), which have positive exo- and endo-symbiotic association with methanogens, thus lowering protozoa-associated methanogens and their activities (Patra and Saxena, 2009); propynoic acid is directly toxic to methanogens (Zhou et al., 2011); and nitrate may serve as competitive electron acceptors diverting hydrogen away from methanogenesis and directly inhibit methanogens in the form of nitrite (Bozic et al., 2009; Zhou et al., 2012). Thus, it was hypothesized that these inhibitors may act additively and/or synergistically in inhibiting different groups of microbes involved in methane production when used in combinations, thus achieving effective reduction of methane production at low concentrations that do not adversely affect feed digestion or fermentation. Therefore, the objective of this study was to explore possible combinations of quillaja saponin, propynoic acid, and nitrate in inhibiting methane production, and their effects on feed digestion, rumen fermentation, diversity of bacteria and archaea, and abundances of major cellulolytic bacterial populations using an *in vitro* model.

2. Methods

2.1. Experimental design

Quillaja saponin (from the bark of *Quillaja saponaria* Molina plants) and propynoic acid (95% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and sodium nitrate (also from Sigma–Aldrich) was used as a source of nitrate. Quillaja saponin, propynoic acid, and nitrate were used at two different doses individually or in their two- and three-way combinations. There were 15 treatments in total: control (without any methanogenic inhibitor), saponin at low (0.6 g/L; SL) and high (1.2 g/L; SH) doses, propynoic acid at low (4 mM; PL) and high (8 mM; PH) doses, nitrate at low (5 mM; NL) and high (10 mM; NH) doses, and combinations of the three inhibitors (Table 1). The doses of these compounds were selected based on previous studies (Ungerfeld et al., 2007; Zhou et al., 2011; Patra et al., 2012). Each of these treatments was evaluated in three replicates. The sapogenin content of the quillaja saponin was 24.2%, which was determined by the gravimetric method after acid hydrolysis of glycosidic linkages in saponin in a previous study (Patra et al., 2012).

2.2. Inoculum preparation and incubation

Fresh rumen fluid was collected from two cannulated lactating Jersey cows at approximately 10 h post morning feeding. The cows were fed a total mixed ration (TMR) that was composed (% dry matter (DM) basis) of corn silage (45%), alfalfa hay (10%), Cargill dairy protein product (20%), and a concentrate mixture (25%). The concentrate mixture consisted of 46.8% ground shelled corn, 12.2% soybean meal, 30.3% AminoPlus® (Ag Processing Inc., Omaha, NE), 1.8% tallow, 2.2% salt, 4.3% limestone, 0.11% magnesium oxide, and vitamin and trace minerals. The cows had free access to the TMR that was delivered twice a day at 5:30 am and 4:00 pm. The rumen fluid collected from each of the cows was mixed equally, and then filtered through three layers of sterile cheesecloth for use as the inoculum in the *in vitro* batch rumen fermentation.

The *in vitro* batch fermentation was carried out in 120-mL serum bottles as described in previous studies (Patra and Yu, 2012, 2013a). The feed substrate for the *in vitro* fermentation was a mixture of alfalfa hay and a dairy concentrate feed at the ratio of 50:50. The concentrate feed consisted mainly of ground corn (33.2%), soybean meal (14.2%), AminoPlus® (15.5%), distillers grains (19.8%), and wheat middlings (11.3%). The buffered medium for the *in vitro* rumen fermentation was prepared anaerobically according to the procedure of Menke and Steingass (1988). The anaerobic buffered medium (30 mL) and the rumen inoculum (10 mL) were dispensed into each serum bottle containing 400 mg of ground feed substrate in an anaerobic chamber. The headspace of these bottles contained carbon dioxide only. These serum bottles were each sealed with a butyl rubber stoppers and then incubated at 39 °C for 24 h in a water bath with intermittent shaking.

Table 1
Combinations of methanogenic inhibitors used in this study.

Treatment	Quillaja saponin (g/L)	Sodium nitrate (mM)	Propynoic acid (mM)
SL + NL	0.6	5	0
SL + PL	0.6	0	4
PL + NL	0	5	4
SH + NH	1.2	10	0
SH + PH	1.2	0	8
PH + NH	0	10	8
SL + NL + PL	0.6	5	4
SH + NH + PH	1.2	10	8

2.3. Sampling and measurements

After 24 h incubation, gas pressure in each of the bottles was measured using a manometer (Traceable®; Fisher Scientific, USA) to determine total gas production. Then 10 mL of each headspace gas was collected into a glass tube pre-filled with distilled water by displacement. The culture samples (1 mL) were collected in microcentrifuge tubes for microbial analysis. The pH values of the fermentation media were recorded using a pH meter (Fisher Scientific, USA), and then the cultures were filtered through filter bags (ANKOM Technology, USA) to determine degradability of feeds. The filtrates were sampled into 2 mL microfuge tubes for VFA (1 mL) and ammonia analyses (2 mL). All the samples were stored at −20 °C until further processing. The samples for VFA analysis were added with equal volume of 33% metaphosphoric acid and were then centrifuged (16,100×*g* for 10 min at 4 °C). The supernatants (0.5 mL) were pipetted into 2-mL clear crimp glass vials (Suppelco, Bellefonte, USA), which were sealed with caps (Fisher Scientific, USA) after adding an internal standard (50 μL of 0.2% 2-ethylbutyric acid) and stored at 4 °C until gas chromatography analysis.

The concentrations of methane in the gas samples were determined using a gas chromatograph (HP 5890 Series, Agilent Technologies, USA) equipped with a thermal conductivity detector and a HP-PLOT Q capillary column coated with porous polymer particles made of divinylbenzene and ethylvinylbenzene (Agilent Technologies Inc., USA). The VFA concentrations in fermentation media were analyzed using a gas chromatograph (HP 5890 series, Agilent Technologies, USA) fitted with a flame ionization detector. The concentrations of ammonia in the fermentation media were measured by calorimetric method (Chaney and Marbach, 1962).

The DM of the feed substrates and undegraded residues in filter bags was determined after drying at 105 °C in a hot air oven (AOAC, 2007). The concentration of neutral detergent fiber (NDF) in the feed and residues was analyzed by treatment with a neutral detergent solution without α-amylase and addition of sodium sulfite (Van Soest et al., 1991). Apparent DM and NDF degradabilities of the feed substrate were calculated by the difference in amounts