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Combinations of nitrate, saponin, and sulfate additively reduce methane production by rumen cultures *in vitro* while not adversely affecting feed digestion, fermentation or microbial communities

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HIGHLIGHTS

- Nitrate–sulfate–saponin combination additively inhibited methanogenesis (by 46%).
- This combination did not adversely affect feed digestion or fermentation.
- This combination also reduced abundances of protozoa, but not methanogens.
- This combination increased abundances of select cellulolytic bacterial populations.
- Combinations of the compounds altered communities of archaea and bacteria.

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ABSTRACT

This study investigated the effects of saponin (0.6 g/L), nitrate (5 mM) and sulfate (5 mM), alone and in combinations, on methanogenesis, rumen fermentation, microbial community, and abundances of select microbial populations using *in vitro* rumen culture. Combinations of nitrate with saponin and/or sulfate additively suppressed methane production, with the lowest reduction (nearly 46%) observed for the combination of all the three inhibitors. None of the treatments adversely affected feed digestion or rumen fermentation. All the inhibitors, either alone or in combinations, did not alter the abundances of total bacteria, *Ruminococcus albus*, or archaea. However, saponin, alone and together with nitrate and/or sulfate, increased the abundance of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, but decreased that of protozoa. DGGE analyses revealed limited changes in both bacterial and archaeal communities by the treatments. The nitrate–saponin–sulfate combination may be an effective and practical strategy to mitigate methane emission from ruminants.

1. Introduction

Livestock production systems contribute 12–18% to the global anthropogenic greenhouse gas (GHG) emissions in carbon dioxide equivalents (CO_2-eq) (Steinfeld et al., 2006; Westhoek et al., 2011). The direct methane and nitrous oxide emissions from enteric fermentation and manure management practices (including manure application) contribute 5.4 Gt CO_2-eq GHG to the global emissions annually (FAO, 2013). Enteric methane emission is the largest source of GHG from agriculture. This contribution will likely continue to increase over the next few decades due to growing demands for meat and milk primarily driven by human population growth and improved standard of living in developing countries (Patra, 2014). Concerns over the significant contribution from livestock farming to global warming have spurred numerous studies aiming to advance the scientific knowledge on GHG emissions by livestock at global, national, and local levels (Bellarby et al., 2013; Gerber et al., 2011) and to develop various strategies to mitigate GHG emissions from livestock, especially cattle (Bellarby et al., 2013; Patra, 2012).

Many types of methane inhibitors have been repeatedly tried, primarily individually, to lower enteric methane production by ruminants (Patra, 2012). However, each of them often exerts adverse effects on feed digestion and rumen fermentation when added at high enough doses to achieve effective methane inhibition (Patra and Yu, 2013a). In addition, some of these inhibitors are toxic to animals (Patra, 2012). The aforementioned adverse

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and toxic effects can be avoided at low doses, but unfortunately inhibition to methane emission diminishes also. To address this dilemma, it was hypothesized that combinations of anti-methanogenic inhibitors with complementary mechanism of actions may synergistically or additively decrease methane production without any adverse effects on feed digestion or fermentation at low doses (Patra and Yu, 2013a). In that study (Patra and Yu, 2013a), a combination of nitrate and quillaja saponin was shown to reduce methane production dramatically (by 32% at 5 mM nitrate and 0.6 g/L saponin; and by 58% at 10 mM nitrate and 1.2 g/L saponin) using an *in vitro* model of rumen cultures (Patra and Yu, 2013a). In the nitrate–saponin combination, three modes of action were shown to function additively in reducing methane production: (1) quillaja saponin functioning as an inhibitor to rumen protozoa, decreasing hydrogen production by protozoa and protozoa-associated methanogens (Patra and Saxena, 2009), (2) nitrate acting as an electron sink and competing with CO_2 for electrons, and (3) nitrite, the first intermediate of nitrate reduction, exerting toxicity to methanogens (Bozic et al., 2009; Zhou et al., 2011).

Dissimilatory sulfate reduction by sulfate reducing bacteria (SRB) is thermodynamically more favorable ($\Delta G_0 = -42.2$ kJ/mol H_2) than hydrogenotrophic methanogenesis ($\Delta G_0 = -33.9$ kJ/mol H_2) (Ungerfeld and Kohn, 2006). Indeed, previous studies have shown that sulfate outcompeted CO_2 as an electron acceptor in anaerobic habitats (Lovley et al., 1982; Muyzer and Stams, 2008). One recent study has shown that sulfate can suppress methane production in sheep (van Zijderveld et al., 2010). It was thus hypothesized that combinations of nitrate, sulfate, and saponin may further reduce methane production by rumen microbial communities than the combination of nitrate and saponin. Therefore, the objective of this study was to test this hypothesis and evaluate the effect on feed digestion, fermentation, communities of bacteria and archaea, and abundances of select cellulolytic bacterial populations. The results may guide future research and development of effective and practical strategies to mitigate methane emission from cattle.

2. Methods

2.1. Experimental design

Quillaja saponin (from the bark of *Quillaja saponaria* Molina plants) was purchased from Sigma–Aldrich (St. Louis, MO, USA); sodium nitrate and potassium sulfate was used as sources of nitrate and sulfate, respectively. The sapogenin content in the quillaja saponin product was 24%. Quillaja saponin (0.6 g/L), nitrate (5 mM), and sulfate (5 mM) were used individually or in two- and three-way combinations, resulting in 8 treatments: control (without any methanogenic inhibitor), saponin, nitrate, sulfate, nitrate plus saponin, nitrate plus sulfate, sulfate plus saponin, and nitrate plus sulfate and saponin.

2.2. Preparation of medium, inoculum, and incubation

Preparation of the buffered medium, inoculum, and *in vitro* incubation was performed essentially as described previously (Patra and Yu, 2013b). Briefly, fresh rumen fluid obtained from two cannulated lactating Jersey cows at around 10 h post morning feeding was used as the inoculum. The two cows were fed a total mixed ration (TMR) composed (% dry matter (DM) basis) of corn silage (33%), alfalfa hay (10%), Cargill dairy protein product (30%), and a concentrate mixture (27%). The rumen fluid collected from each of the two cows was mixed equally and then filtered through three layers of sterile cheesecloth before inoculation. The *in vitro* batch fermentation was carried out in 120-ml serum bottles in triplicates (Patra and Yu, 2012, 2013a). The feed substrate was a mixture of alfalfa hay and a dairy concentrate feed at a 50:50 ratio. 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* fermentation was prepared anaerobically (Menke and Steingass, 1988), and 30 ml of the medium and 10 ml of rumen fluid (the inoculum) 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 sealed with a butyl rubber stoppers and incubated at 39 °C for 24 h in a water bath with intermittent shaking.

2.3. Sampling and chemical analysis

After 24 h of fermentation, gas pressure in the culture bottles was measured using a manometer (Traceable®; Fisher Scientific, USA) to determine total gas production. Then gas sample was collected from each bottle into a glass tube, which was pre-filled with distilled water and sealed with a butyl rubber stopper, by displacement. The gas sample tubes were stored upside down to prevent loss of the gas samples. One milliliter culture was collected from each culture bottle into a microcentrifuge tube for microbial analysis. Then, pH values of the *in vitro* cultures were immediately recorded using a pH meter (Fisher Scientific, USA). The remaining content of each culture bottle was filtered through a filter bag (ANKOM Technology, USA) to determine degradability of the feed substrate. The filtrates were sampled into microcentrifuge tubes for analysis for volatile fatty acids (VFA) and ammonia. All the samples were stored at −20 °C until further analyses.

The concentrations of methane in 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 concentrations of each VFA were also analyzed using a gas chromatograph (HP 5890 series, Agilent Technologies, USA) fitted with a flame ionization detector and a Chromosorb W AW packed glass column (Sigma–Aldrich, USA). The concentrations of ammonia in the fermentation media were determined colorometrically (Chaney and Marbach, 1962). The degradabilities of DM and neutral detergent fiber (NDF) of the substrate were determined gravimetrically (Blümmel et al., 1997).

2.4. DNA extraction, qPCR, and denaturing gradient gel electrophoresis (DGGE)

Metagenomic DNA was extracted from each culture sample following the procedure described by Yu and Morrison (2004a). The DNA quality was evaluated using agarose gel (1%) electrophoresis, and DNA yield was quantified using the Quant-iTdsDNA Broad Range Assay kit (Invitrogen Corporation, Carlsbad, CA, USA) on a Stratagene Mx3000p machine (La Jolla, CA, USA). The DNA samples were stored at −20 °C until analyses.

The population sizes of total archaea, total protozoa, and select bacterial species were quantified using SYBR Green-based qRT-PCR using a Stratagene Mx3000p machine following the procedure described earlier (Patra and Yu, 2013b). Briefly, the sample-derived qPCR standards were prepared using the respective specific PCR primer sets and a composite DNA sample that were prepared by pooling an equal amount of all the metagenomic DNA samples (Yu et al., 2005; Patra and Yu, 2013b). The standards were then purified using a PCR Purification kit (Qiagen, USA) and quantified. For each of the standards, 16S rRNA (*rrs*) gene copy numbers were calculated based on the length of the PCR products and the mass concentrations (Yu et al., 2005). Tenfold serial dilutions were prepared in Tris–EDTA buffer prior to qRT-PCR assays. To minimize