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Effects on enteric methane production and bacterial and archaeal communities by the addition of cashew nut shell extract or glycerol—An in vitro evaluation

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ABSTRACT

The objective of the study was to evaluate the effect of cashew nut shell extract (CNSE) and glycerol (purity >99%) on enteric methane (CH_4) production and microbial communities in an automated gas in vitro system. Microbial communities from the in vitro system were compared with samples from the donor cows, in vivo. Inoculated rumen fluid was mixed with a diet with a 60:40 forage:concentrate ratio and, in total, 5 different treatments were set up: 5 mg of CNSE (CNSE-L), 10 mg of CNSE (CNSE-H), 15 mmol of glycerol/L (glycerol-L), and 30 mmol of glycerol/L (glycerol-H), and a control without feed additive. Gas samples were taken at 2, 4, 8, 24, 32, and 48 h of incubation, and the CH_4 concentration was measured. Samples of rumen fluid were taken for volatile fatty acid analysis and for microbial sequence analyses after 8, 24, and 48 h of incubation. In vivo rumen samples from the cows were taken 2 h after the morning feeding at 3 consecutive days to compare the in vitro system with in vivo conditions. The gas data and data from microbial sequence analysis (454 sequencing) were analyzed using a mixed model and principal components analysis. These analyses illustrated that CH_4 production was reduced with the CNSE treatment, by 8 and 18%, respectively, for the L and H concentration. Glycerol instead increased CH_4 production by 8 and 12%, respectively, for the L and H concentration. The inhibition with CNSE could be due to the observed shift in bacterial population, possibly resulting in decreased production of hydrogen or formate, the methanogenic substrates. Alternatively the response could be explained by a shift in the methanogenic community. In the glycerol treatments, no main differences in bacterial or archaeal population were detected compared with the in vivo control. Thus, the increase in CH_4 production may be explained by the increase in substrate in the in vitro system. The reduced CH_4 production in vitro with CNSE suggests that CNSE can be a promising inhibitor of CH_4 formation in the rumen of dairy cows.

Key words: in vitro, methane production, cashew nut shell extract, glycerol

INTRODUCTION

The continuous increase in methane (CH_4) concentrations in the atmosphere has been an important issue over the last few decades, as CH_4 is one of the major greenhouse gases and consequently contributes to climate change (Beauchemin et al., 2008). Ruminants contribute considerably to this (e.g., 28% of total anthropogenic CH_4 in the United States comes from cattle; Gerber et al., 2013). The production of CH_4 from ruminants also causes energy losses for the animal, corresponding to 2 to 12% of gross energy (**GE**) intake (Johnson and Johnson, 1995). Methane is produced in the rumen by methanogens (Archaea), which mainly use hydrogen and CO_2 as their energy and carbon source, respectively. These compounds are mainly released during the degradation of carbohydrates and, to a lesser extent, amino acids to VFA by different fermentative microorganisms. In addition to hydrogen-, formate-, and methyl-containing compounds, fermentation end products also are important methanogenic substrates in the rumen (Hungate et al., 1970; Leahy et al., 2013). The amounts and types of feeds consumed by the cow appear to have the biggest effect on the amount of CH_4 produced (Johnson and Johnson, 1995; Ramin and Huhtanen, 2013), but other factors also exist, such as individual variation, breed, and geographical location (Janssen and Kirs, 2008; King et al., 2011; Danielsson et al., 2012). Some successful strategies to inhibit CH_4 production involve feeding of supplements, such as feedstuffs high in lipids, ionophores, plant compounds, and enzymes (Beauchemin et al., 2009; Hook et al., 2010).

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Feeding supplements as cashew (*Anacardium occidentale*) nut shell extract (**CNSE**) and glycerol has been tested in previous studies, with observed inhibition effects on CH_4 production (Watanabe et al., 2010; Lee et al., 2011; Ramin et al., 2011; Shinkai et al., 2012). Currently, the effect of CNSE on CH_4 production is unclear and may depend on a direct inhibition effect of Archaea or an indirect effect due to decreased bacterial production of methanogenic substrates (i.e., hydrogen or formate). Furthermore, there is a lack of knowledge concerning the effects of glycerol on the microbial communities and in relation to CH_4 production.

Cashew nut shell liquid is a by-product from the cashew nut industry and the global production is estimated at 450,000 t/yr (Patel et al., 2006); the price today is \$400 to \$600/t (Alibaba, 2014). The product CNSE is produced by a cold extraction procedure as has been described by Philip et al. (2007). Cashew nut shell extract mainly contains the phenolic constituents of anacardic acid, cardol, and cardanol. Anacardic acid has antimicrobial activity and it is suggested to selectively inhibit certain gram-positive rumen bacteria (Kubo et al., 2003). The nut shell can also be heat treated, but the resulting products have comparably low concentrations of anacardic acid and the antimethanogenic activity is destroyed (Watanabe at al., 2010). In vivo studies in cow rumina (CNSE fed in a pellet form) and studies for in vitro batch cultivation (CNSE liquid added to the rumen fluid) have shown that CNSE inhibited CH_4 production by between approximately 20 and 60% (Watanabe et al., 2010; Ramin et al., 2011; Shinkai et al., 2012). Furthermore, when CNSE was added to pure cultures of different bacteria, a restricted effect on growth of hydrogen-, formate-, and butyrate-producing bacteria was observed (Watanabe et al., 2010). However, even though CH_4 production was greatly reduced, the relative abundance of Archaea was not affected by CNSE.

The biodiesel industry is currently producing, as a by-product, large quantities of glycerol (synonym: glycerin or 1,2,3-propanetriol), and the annual European production has been estimated to be 11.2 million tonnes in 2010 (Khanna et al., 2012). Glycerol is a potential high-energy feed additive for cows. The price of glycerol depends on its purity and, at present, the price of refined glycerol (99%) in Sweden is around €0.6/kg. Glycerol has been shown to affect VFA profiles (i.e., increased concentrations of propionate with unaffected concentrations of acetate) in cows (DeFrain et al., 2004), steers (Wang et al., 2009), and sheep (Schröder and Südekum, 1999). Moreover, Lee et al. (2011) showed that the addition of glycerol to an in vitro incubation reduced CH_4 production.

In vitro systems [e.g., continuous-culture experiments as described by Czerkawski and Breckenridge (1977) and batch culture experiments as reported by van Nevel and Demeyer (1981)] are commonly used for evaluating the effects of diets and additives on enteric CH_4 production. The advantages of an in vitro system compared with in vivo are the reduced effect on the animal, lower costs, and it is also likely easier to standardize. However, the limitations of the in vitro system have to be considered before conclusions can be transferred to the in vivo situation. One important aspect is that rumen VFA in cows is continuously absorbed through the rumen wall, maintaining the concentration of VFA at a similar level. In the in vitro system, VFA might accumulate over time during fermentation. Such an accumulation could theoretically have an effect on the development of the microbial population and, consequently, also on CH_4 production.

The aim of this experiment was to investigate the effects of CNSE and glycerol on the in vitro production of CH_4 and VFA and to investigate effects of these feed additives on the archaeal and bacterial community structures.

MATERIALS AND METHODS

All management of animals was approved by the Umeå Ethical Committee for Animal Research (Umeå, Sweden).

Animals

Three dairy cows of the Swedish Red breed at late lactation, fed a diet with a silage:concentrate ratio of 600:400 g/kg of DM were included in the experiment. Rumen fluid was collected 2 h after the morning feed and fluid from each cow was strained separately through a double layer of cheesecloth into prewarmed thermos flasks that had previously been flushed with CO_2. The rumen fluid was transported to the laboratory within 10 min of collection and pooled in equal amounts. The pH was measured and a 1-mL subsample was taken and stored at −20°C for further analysis of VFA and microbial community structure. Rumen fluid was strained through 4 layers of cheesecloth and mixed with buffered mineral solution (20:80, vol/vol; Menke and Steingass, 1988) supplemented with peptone (pancreatic digested casein; Merck KGaA, Darmstadt, Germany) at 39°C under constant stirring and continuous flushing with CO_2.

Experimental Design and Laboratory Procedures

Prior to the in vitro incubation, samples were dried at 60°C for 48 h and further ground through a 1.0-mm