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Attenuation of Ruminal Methanogenesis

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ATTENUATION OF RUMINAL METHANOGENESIS

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Ruminal methanogens reduce carbon dioxide to methane (CH_4), thereby preventing hydrogen use by bacteria for VFA synthesis resulting in a 2 to 12% loss in feed gross energy. Methane is a greenhouse gas that contributes to global warming. The objectives of this work were to determine: 1) the extent to which ruminal cultures acquire resistance to a nitrofuranyl derivative of para-aminobenzoate (NFP) and an extract from the plant *Yucca shidigera* (Yucca); 2) the effect of distillers dried grains plus solubles (DDGS) on ruminal CH_4 production; 3) the effect of brome hay-based diets, corn-based diets, and in vivo 2-bromoethansulfonate treatment on ruminal methane (CH_4) production; and 4) the effect of the above treatments on the methanogen population. Ruminal cultures treated with NFP for 90 d maintained a diminished capacity to generate CH_4, but cultures became resistant to the inhibitory effects of Yucca treatment within 10 d. Both treatments decreased ($P < 0.01$) the relative abundance of total Archaea and the order Methanomicrobiales, but Yucca treatment increased ($P < 0.01$) the relative abundance of the order Methanobacteriales. The replacement of brome hay and corn with DDGS in lamb diets decreased ($P < 0.01$) and increased ($P < 0.05$), respectively, the amount of CH_4 produced per unit of digested DM. The substitution of DDGS for brome hay increased ($P < 0.01$) the relative abundance of the order Methanomicrobiales. The replacement of brome hay with corn decreased ($P < 0.05$) the amount of CH_4 produced per unit of digested DM, and also decreased ($P < 0.05$) the relative abundance of both

Archaea and the order Methanomicrobiales. However, the abundance of the order Methanobacteriales increased ($P < 0.05$) as corn replaced brome hay. Intraruminal administration of 2-bromoethansulfonate decreased ($P < 0.05$) CH_4 emissions, and decreased ($P < 0.05$) the relative abundance of Archaea and Methanobacteriales. In conclusion, NFP may be efficacious for chronically inhibiting ruminal methanogenesis, and the replacement of dietary forage with DDGS attenuates CH_4 emissions from ruminant animals. Changes in domain- and order-specific ribosomal DNA indicators of methanogens are not consistently correlated with changes in CH_4 production.

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CHAPTER I

Review of Literature

Attenuation of Ruminal Methanogenesis

Methane of Ruminal Origin

Ruminal fermentation. Ruminal fermentation is advantageous to the host because it facilitates the catabolism of feedstuff components that are otherwise indigestible by the host animal. A negative consequence of fermentation is the production of (**CH_4**). The rumen provides an environment that: is moist, anaerobic, and well buffered; has a relatively constant influx of substrates and efflux of products; and has a carefully regulated temperature (Hungate, 1988; Yokoyama and Johnson, 1988; Johnson et al., 2000; Russell, 2002). These conditions provide an ideal habitat for the proliferation of anaerobic microorganisms. It allowed for the evolutionary development of a symbiotic relationship between ruminal microbes and the ruminant animal, in which the ruminant animal benefits from many of the products of microbial metabolism (Hungate, 1988; Yokoyama and Johnson, 1988; Russell, 2002).

The ruminal microflora consist of bacteria (10^{10}-10^{11} cells/g), bacteriophages (10^{7}-10^{9} particles/g), protozoa (10^{4}-10^{6} cells/g), fungi (10^{2}-10^{4} cells/g), and methanogenic archaea (10^{9}-10^{10} cells/g) (Joblin, 2005; McSweeney et al., 2005). Some of the bacteria, protozoa, and fungi possess the enzymes required to hydrolyze the linkages between residues of structural carbohydrates of plants into sugars (Yokoyama

and Johnson, 1988; Johnson et al., 2000). Sugars, along with plant cell solubles, are then further catabolized to VFA, which are absorbed by the host animal and used to ultimately generate ATP (Owens and Goetsch, 1988; Johnson et al., 2000; Russell, 2002). The VFA produced in the largest quantities are acetate, propionate, and butyrate (Figure 1.1) (Owens and Goetsch, 1988; Russell, 2002). Carbon dioxide (**CO_2**) is a byproduct of the reactions that generate the VFA with an even number of carbons, or propionate and acetate via the direct reductive pathway (Russell, 2002). Furthermore, both the catabolism of sugars to pyruvate and the production of 2 acetyl CoA molecules from 2 pyruvate molecules results in a net accumulation of NADH and $FADH_2$ (Figure 1.1). The oxidized forms of these cofactors must be regenerated to continue the catabolism of sugars to VFA, and because O_2 is not present to serve as a terminal electron acceptor an alternative must be utilized. Two methods utilized for the disposal of reducing equivalents are the production of the more reduced VFA and the production of H_2 by membrane bound hydrogenases (Russell, 2002). However, these hydrogenases have an acute sensitivity to an increased partial pressure of H_2. Therefore, the role of methanogens in the rumen is to scavenge H_2 and keep the partial pressure of H_2 low enough for the hydrogenases to function (Russell, 2002).

The relative amounts of VFA, CO_2, and CH_4 produced are a function of several factors, many of which are not yet completely understood, but may be predicted. Wolin (1960) published an equation that allowed for an estimation of the amount of CO_2 and CH_4 produced by ruminal fermentation based on the concentration of VFA. Because

glucose has an oxidation-reduction state (redox) of 0, the redox of the sum of all of the products of fermentation must also equal 0. Wolin's equation is:

$$([\text{Acetate}] \times 0) + ([\text{Propionate}] \times -1) + ([\text{Butyrate}] \times -2) + ([CO_2] \times 2) + ([CH_4] \times -2) = 0. \quad [1.1]$$

Therefore, according to Wolin's fermentation balance, an increase in the production of the more reduced VFA will be associated with decreased CH_4 production, but an increase in the production of the more oxidized VFA will be associated with an increase in CH_4 production. The limitations of Wolin's fermentation balance include: 1) only carbohydrates of the empirical formula $C_6H_{12}O_6$ are accommodated, and 2) bacterial cell wall lipids, hydrogen, and other end-products are not considered (Russell, 2002). As opposed to using end-products to calculate CH_4 production, other researchers have utilized dietary variables to predict CH_4 emissions. Blaxter and Clapperton (1965) used CH_4 emission data from several experiments with sheep and cattle, and calculated CH_4 production (as a percentage of GE) to be a function of digestibility (D) and intake level (L) with the equation:

$$CH_4 = 1.30 + (0.112 \times D) - (L \times (2.37 – (0.05 \times D)) \quad [1.2]$$

Moe and Tyrrell (1979) reported that CH_4 production (Mcal/day) may be predicted based on the amount and type of carbohydrate ingested utilizing the equation:

$$CH_4 = 0.814 + (0.122 \times \text{soluble residue}) + (0.415 \times \text{hemicellulose}) + (0.633 \times \text{cellulose}) \quad [1.3]$$

Aside from a few inherent exceptions, the results from all of these estimation tools follow the same theme of diets resulting in a decreased acetate:propionate ratio yield less CH_4 than those that result in a greater acetate:propionate ratio.

Acetogenic bacteria in the hindgut of mammals and termites are capable of reducing CO_2 to produce acetate ($2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$) (Ljungdahl, 1986). The concentration of these bacteria in the rumen is similar to that of methanogens (Leedle and Greening, 1988). However, the formation of acetate in the rumen from radio-labeled CO_2 was not observed by Prins and Lankhorst (1977). The absence of reductive acetogenesis can likely be attributed to the ability of this group of bacteria to utilize substrates other than H_2 for their energy supply (Moss et al., 2000) and also to these bacteria's lesser affinity for H_2 relative to methanogens (Russell, 2002). Perhaps inhibition of methanogens could increase the ruminal partial pressure of H_2 enough to stimulate the production of acetate from CO_2 by this group of bacteria.

Methanogenesis and its relationship with feed efficiency. A negative implication of ruminal CH_4 production is that the carbon and reducing equivalents comprising each molecule are eructated from the ruminal environment resulting in a direct loss of feed GE. Estimates for the percentage of feed GE lost as CH_4 range from 2 to 12% and this value can be affected by a variety of factors including DMI, feed processing, type of carbohydrate, dietary fat, and any compounds that specifically affect methanogenesis (Johnson and Johnson, 1995). The fractional amount of feed GE lost as CH_4 decreases as DMI increases (Blaxter and Clapperton, 1965; Moe and Tyrrell, 1979; Johnson and Johnson, 1995; Benchaar et al., 2001). This effect of increasing intake on

fractional CH_4 losses is more pronounced in diets that have a greater digestibility (concentrate-based diets) compared to those that are less digestible (forage-based diets) in the rumen (Blaxter and Clapperton, 1965; Benchaar et al., 2001). Therefore, limited intake of a highly digestible feed will result in a greater fractional CH_4 loss than will high intake of the same feed. Johnson and Johnson (1995) report that fractional losses of GE decrease 1.6% per level of intake. Forage processing has also been reported to affect methanogenesis with grinding (Johnson and Johnson, 1995) and pelleting (Johnson and Johnson, 1995; Hironaka et al., 1996; Benchaar et al., 2001) decreasing CH_4 production. However, this effect is not observed when intakes are restricted (Johnson and Johnson, 1995). The effects of carbohydrate type, dietary fat, and methanogenesis inhibitory compounds on ruminal CH_4 production are elaborated upon later in this review. Because some feeding regimens reduce fractional losses of feed GE to CH_4, strategic utilization of these regimens may further increase the efficiency of ruminal fermentation.

Methane is a greenhouse gas. Solar energy radiated in the visible part of the spectrum (0.4 to 0.7 µM) warms the earth's surface, as much of it passes through the atmosphere without being absorbed (Moss, 1993; Moss et al., 2000). After warming the earth's surface the energy is radiated back from the surface in the infra-red spectrum (4 to 100 µM), with the majority (approximately 70%) passing back through the atmosphere into space (Moss, 1993; Moss et al., 2000). The remaining infra-red radiation is absorbed primarily by CO_2 and H_2O molecules in the atmosphere, which in turn radiate the energy back to earth to assist in maintaining a relatively constant temperature (Moss, 1993; Moss et al., 2000). Therefore, increased atmospheric concentrations of CO_2, or other gases

capable of absorbing infra-red radiation, increases the amount of energy radiated back to the earth, which has been termed the greenhouse effect.

Since the pre-industrial era (1750), atmospheric CO_2, CH_4, and NO_2, have increased by 35, 143, and 18 percent, respectively, and much of this increase is attributed to anthropogenic sources (IPCC, 2001). Carbon dioxide, CH_4, and NO_2 are the 3 most important greenhouse gases based on their potency and abundance. Relative to CO_2, CH_4 and NO_2 have a global warming potential (**GWP**), which is calculated based on the radiative force of each gas relative to CO_2, of 21 and 310, respectively (EPA, 2007). Utilizing the GWP and the gross emissions of each gas, EPA (2007) reported greenhouse gas emissions from anthropogenic sources in weighted units of Tg CO_2 equivalents (Table 1.1). Anthropogenic CO_2 emissions are far greater than that of any other greenhouse gas and have continued to increase over the past 15 years. Total anthropogenic CH_4 emissions are < 10% that of CO_2 emissions and are no longer increasing. Landfills are responsible for the greatest CH_4 emissions followed by enteric fermentation. Methane from enteric fermentation includes that occurring in the foregut (ruminant animals) and hindgut (ruminant and monogastric animals), with the former predominating. Beef cattle are responsible for the greatest amount of CH_4 of enteric origin and about 75% of this CH_4 is from cow-calf operations (Chase, 2006). Dairy cattle are responsible for approximately 25% of enteric CH_4 with the balance arising from horses, sheep, swine, and goats.

Compared to the combustion of fossil fuels, which results in 94% of CO_2 and 79% of total greenhouse gas emissions from anthropogenic sources (EPA, 2007), the

contribution that ruminal CH_4 production makes toward total greenhouse gas emissions is minimal (1.5%). However, the atmospheric half-life of CH_4 (9 to 15 yr) is much less than that of CO_2 or NO_2 (50-200 or 120 yr, respectively)(EPA, 2007). Therefore, reducing the amount of CH_4 resulting from ruminal fermentation will not reverse the long-term trend of global warming but does present an opportunity to provide short-term alleviation from the greenhouse effect.

Methanogens

Archaea. Methanogens are methane-producing anaerobes and make up the largest group within the domain Archaea (Ferry and Kastead, 2007). All living organisms were divided by 1 of 2 systems prior to the proposal (Woese et al., 1990), and subsequent acceptance by the majority of the scientific community, for a new taxon above the level of kingdom called “domain”. These two systems were the five-kingdom taxonomy (Animalia, Plantae, Fungi, Protista, and Monera) or the eukaryote-prokaryote dichotomy. A pitfall common to both classification schemes was that Bacteria and Archaea were classified similarly (Monera or Prokaryotes), as they do share the common characteristic of lacking membrane bound organelles. However, this classification scheme was refuted as molecular techniques allowed for the sequencing of the 16S rRNA gene, revealed the true lineage of these microorganisms, and allowed for the definitive establishment of the domains Archaea, Bacteria, and Eucarya (Woese et al., 1990). The taxonomic classification and lineage of seven species of methanogenic archaea that have been isolated (Joblin, 2005) and the four orders that have been detected with DNA hybridization (Lin et al., 1997) from ruminal contents are indicated in Figure 1.2.

Ruminal methanogens. Because the foregut of ruminant animals provides an environment that meets the fastidious growth requirements of certain species of methanogenic archaea, ruminal methanogens have been an integral part of the evolution of the resident microflora (Hungate, 1988; Yokoyama and Johnson, 1988; Johnson et al., 2000; Russell, 2002). Methanogens are found in a symbiotic association with ruminal bacteria (Wolin and Miller, 1988) and protozoa (Lange et al., 2005). These ruminal microorganisms utilize the CO_2 and H_2 produced by the protozoa and bacteria from the catabolism of hexoses to produce CH_4 and generate ATP (Ferry and Kastead, 2007; Albers et al., 2007), which benefits the donors by providing an electron sink for reducing equivalents to minimize the partial pressure of H_2 (Wolin and Miller, 1988; Russell, 2002; Lange et al., 2005).

Identification of methanogens. Culture-based techniques have allowed for the isolation and identification of a limited number of species of ruminal methanogens from the orders Methanobacteriales, Methanomicrobioles, and Methanosarcinales (Joblin, 2005). Because of methanogens' fastidious growth requirements, it not surprising that the utilization of molecular techniques resulted in the discovery of several uncultured species of ruminal methanogens. The true diversity of methanogens present in the rumen has been recently revealed with techniques such as DNA hybridization, the development of clone libraries of 16s rRNA gene sequences, quantitative real-time PCR, and temporal temperature gradient gel electrophoresis (Lin et al., 1997; Sharp et al., 1998; Tokura et al., 1999; Jarvis et al., 2000; Yanagita et al., 2000; Whitford et al., 2001; Tajima et al., 2001; Wright et al., 2004; Skillman et al., 2006; Nicholson et al., 2007; Wright et al.,

2007). Both Lin et al. (1997) and Skillman et al. (2006) utilized DNA hybridization probes to study populations of methanogens, whereas Lin et al. (1997) were the first to report the presence of Methanococcales in the rumen, which had not yet been cultured. Whitford et al. (2001) then reported that the diversity of methanogenenic archaea in the rumen was much more elaborate than expected, and new probes and assays would be required to quantify the extent and composition of this diverse population. The results of these two studies stimulated a flurry of activity that is ongoing and has resulted in the identification of several uncultured, and also not yet identified, species of ruminal methanogens.

Reports of ruminal methanogenic populations, as identified with molecular techniques, indicate the presence of species from four of the five different orders of methanogens from the classes Methanobacteria, Methanomicrobia, and Methanococci (Lin et al., 1997; Sharp et al., 1998; Tokura et al., 1999; Jarvis et al., 2000; Yanagita et al., 2000; Tajima et al., 2001; Skillman et al., 2006; Wright et al., 2007). Many investigators have reported that the most prevalent species in the rumen are the order Methanobacteriales (Sharp et al., 1998; Tokura et al., 1999; Skillman et al., 2006; Nicholson et al., 2007; Wright et al., 2007). Others have reported that species of the order Methanomicrobiales are the most (Yanagita et al., 2000; Tajima et al., 2001) or second most (Sharp et al., 1998; Wright et al., 2007) prevalent in the ruminal environment. There have also been reports of the orders Methanosarcinales (Lin et al., 1997; Jarvis et al., 2000; Nicholson et al., 2007; Wright et al., 2007) and Methanococcales (Lin et al., 1997), but these methanogens were not abundant relative to

other species and the order Methanosarcinales was not found in the rumen of sheep (Lin et al., 1997). The population of methanogens may be affected by the abundance of protozoa. The order Methanbacteriales is associated with ruminal ciliates (Sharp et al., 1998; Tokura et al., 1999) and the order Methanomicrobiales is free-living (Sharp et al., 1998). Because CH_4 production is affected by the quantity and composition of feedstuffs consumed, it is a reasonable assumption that the methanogen population will likewise be affected. Lin et al. (1997) collected samples from different species of animals consuming different diets but the experiment was not designed, nor were the data analyzed, in such a way to detect differences that may have existed between treatments. Independent observations of methanogenic archaea populations in the rumen have been made with grazing cattle (Jarvis et al., 2000; Skillman et al., 2006) and feedlot cattle (Wright et al., 2007), but the effect of diet on the population of methanogens has yet to be elucidated.

Methanogenesis. Methanogens derive energy from the process of methanogenesis with acetate serving as a substrate for approximately two-thirds of all biological CH_4 (Ferry and Kastead, 2007). Reduction of CO_2 with H_2 accounts for another one-third, and a small amount of CH_4 is produced from formate providing electrons and other minor reactions (Ferry and Kastead, 2007). It is thought that the methanogens that utilize acetate grow slowly and are washed out of the rumen, and the majority of ruminal CH_4 is from the reduction of CO_2 (Russell, 2002). Further to this point, the production of CH_4 from acetate is a very slow process and the removal of products from the rumen is too rapid to allow for this to occur (Wolin and Miller, 1988).

Most of the formate within the rumen produced from the production of acetate is converted to CO_2 and H_2 by formate-hydrogen lyase resulting in H_2 being the most likely electron donor for the reduction of CO_2 (Hungate et al., 1970).

The reactants, products, enzymes, and conezymes involved in methanogenesis are well established and identical information is available from several textbooks and review articles. If not otherwise cited, the information in this paragraph can be found in each of four references (Thauer et al., 1993; Deppenmeier, 2002; Shima et al., 2002; Ferry and Kastead, 2007). The first C_1 carrier in methanogenesis is methanofuran, which binds CO_2 and reduces it to formylmethanofuran in a reaction that is catalyzed by formylmethanofuran dehydrogenase (Figure 1.3; Reaction 1). Two separate steps are involved in the initial reduction of CO_2, but formylmethanofuran is the first stable intermediate and it's formation is an indicator of CO_2's commitment to methanogenesis. Tetrahydromethanopterin (**H_4MPT**) is the second C_1 carrier, which receives the formyl group in a reaction catalyzed by formylmethanofuran:H_4MPT formyltransferase (Reaction 2). Tetrahydromethanopterin was initially discovered in archaea (Schworer et al., 1993; Klenk et al., 1997) and it functions similarly to tetrahydrofolate, which serves as a C_1 carrier for most other organisms including non-methanogenic archaea, bacteria, and eucarya (DiMarco et al., 1990; Thauer, 1998). Because of this coezyme's relative uniqueness in most biological systems, it is a prime candidate for the specific inhibition of methanogenesis. Following the formyl group's transfer to H_4MPT, the enzyme methenyl-H_4MPT cyclohydrolase cyclizes the formyl-H_4MPT to methenyl-H_4MPT (Reaction 3). The next reaction is catalyzed by two mechanistically distinct

dehydrogenases. The first enzyme (F_{420}-dependent methylene-H_4MPT dehydrogenase; Reaction 4) oxidizes F_{420} while reducing methenyl-H_4MPT to methylen-H_4MPT. Alternatively, some methanogens utilize an F_{420}-independent enzyme (H_2-forming methylene-H_4MPT dehydrogenase; Reaction 5) that utilizes molecular hydrogen for the reduction of methenyl-H_4MPT. Another F_{420}-dependent enzyme, methylene-H_4MPT reductase, then catalyzes the final reduction that the methyl group will undergo while still attached to H_4MPT (Reaction 6). Methyl-H_4MPT:coenzyme M methyltransferase catalyzes Reaction 7 and this reaction is outlined in detail by Gottschalk and Thauer (2001). Conformational changes in specified subunits of methyl-H_4MPT:coenzyme M methyltransferase, resulting from demethylation (CH_3 transferred from CH_3-H_4MPT) and methylation (CH_3 transferred to CH_3-S-CoM), drives the translocation of sodium (Ferry and Kastead, 2007). Dissipation of the sodium gradient can then be used to generate ATP (Albers et al., 2007). The terminal reaction (Reaction 8) of methanogenesis is catalyzed by the enzyme methyl-coenzyme M reductase (**MCR**), which reduces methyl-coenzyme M with coenzyme B (**CH_3-SCoM** + **HSCoB**) to CH_4 and the heterosulfide of coenzyme M and coenzyme B (**CoMS-SCoB**). A basic understanding of MCR's mechanism of action aids in understanding how the structural analog of coenzyme M, 2-bromoethanesulfonate, inhibits methanogenesis, which is discussed in more detail later in this review. One proposed mechanism for MCR involves the nucleophilic attack of F_{430} on CH_3-S-CoM yielding F_{430}-CH_3 and H-S-CoM intermediates, with subsequent protonolysis of F_{430}-CH_3 resulting in CH4 and the formation of a •S-CoM radical that couples with $^-$S-CoB to form CoM-S-S-CoB (Ferry and Kastead, 2007). Therefore,

replacement of the methyl group of CH_3-S-CoM with another residue can be an effective method of inhibiting MCR (Gunsalus et al., 1978; Balch and Wolfe, 1979b). Lastly, the enzyme heterodisulfide reductase reduces the CoM-S-S-CoB disulfide bond, yielding the active forms of each coenzyme (Reaction 9). As the disulfide bond is reduced, protons are pumped out of the cell to create a proton gradient that results in a proton motive force (Albers et al., 2007) which drives ATP synthesis via an A_1A_0-ATPase protein (Muller et al., 1999).

Anti-methanogenic Compounds

2-bromoethanesulfonate. The compound 2-bromoethansulfonate ($BrCH_2CH_2SO_3^-$; **BES**) is a structural analog of 2-mercaptoethanesulfonate, which interferes with methanogenesis by inhibiting the reductive demethylation of CH_3-S-CoM (Balch and Wolfe, 1979b) and is capable of inhibiting the enzyme at a concentration as low as 10 µM (Gunsalus et al., 1978). 2-bromoethansulfonate is a potent inhibitor of the growth of, and CH_4 production by, pure cultures of methanogens (Smith and Mah, 1978; Balch and Wolfe, 1979a; Balch and Wolfe, 1979b; Smith and Mah, 1981; Smith, 1983; Sparling and Daniels, 1987) yet it has no effect on the growth of a wide variety of eukaryotes (Sparling and Daniels, 1987). The amount of CH_4 produced by mixed cultures of ruminal fluid is also inhibited by BES (Martin and Macy, 1985; Immig et al., 1996; Nollet et al., 1997; Ungerfeld et al., 2004) and VFA production can be stimulated with BES treatment in the presence of a reductive acetogen (Nollet et al., 1997). Therefore, Immig et al. (1996) tested the hypothesis that long-term ruminal infusion of BES may be an effective strategy for chronically inhibiting methanogenesis and

stimulating reductive acetogenesis. A pulse dose (2 g) of BES, followed by continuous infusion (2 g/d) into the rumen of a sheep did decrease ruminal CH_4 production 7 h after the commencement of treatment, but after 3 d of continuous infusion the mixed ruminal microbiota began to acquire resistance to the effects of BES treatment. Furthermore, a pulse dose (2 g) administered 3 d after the termination of infusion did not inhibit CH_4 production.

The ability of ruminal methanogens to adapt to continuous BES treatment was an unforeseen outcome, which instigated an effort to determine the underlying mechanism. Ungerfeld et al. (2004) tested the hypothesis that three different species of methanogens (*Methanobrevibacter ruminantium*, *Methanosarcina mazei*, and *Methanomicrobium mobile*) would differ in their sensitivity to BES. They reported that *Mb. ruminantium* was inhibited by 10, 50, and 250 mM concentrations of BES, *Mm. mobile* was only sensitive to 250 mM BES, and *Ms. mazei* was initially sensitive to 250 mM BES but had begun to acquire resistance between 4 and 6 d of culture. The differential sensitivity to, or acquisition of resistance to, BES observed between species is likely related to interspecies differences in coenzyme M (or its structural analog, BES) transport and metabolism. Resistance to BES is linked to BES uptake (Smith, 1983; Santoro and Konisky, 1987). The inclusion of coenzyme M in the growth medium prevents the uptake of BES (Santoro and Konisky, 1987) and BES inhibition of methanogens is partially relieved when medium coenzyme M concentrations are increased (Balch and Wolfe, 1979a; Smith and Mah, 1981). Ungerfeld et al. (2004) speculated that methanogens that have the ability to synthesize coenzyme M are less dependent on

coenzyme M taken from the medium and will exhibit greater resistance to BES. This speculation is supported by their findings that *Mb. ruminantium* was sensitive to BES and *Ms. mazei* became resistant, because *Mb. ruminantium* requires the presence of extracellular coenzyme M (Taylor et al., 1974) whereas *Ms. mazei* has the ability to synthesize the coenzyme (Stewart et al., 1997). Therefore, the resistance to BES treatment that was observed in vivo (Immig et al., 1996) can likely be attributed to the proliferation of BES-resistant methanogens as opposed to alterations in other ruminal microorganisms and their products. Successful strategies for chronically inhibiting methanogenesis that employ BES will also require a treatment that inhibits the BES-resistant methanogens, which can only be determined after the identification of BES-resistant methanogens that proliferate in vivo.

Analogs of para-aminobenzoic acid. Tetrahydromethanopterin is a coenzyme that is unique to methanogenic archaea and is essential for the reduction of CO_2 to CH_4 (Rouviere and Wolfe, 1988; Graham and White, 2002). H_4MPT serves as an intermediate C_1 carrier through much of methanogenesis (Figure 1.3) and functions similarly to tetrahydrofolate, which has only been discovered in one non-ruminal species of methanogenic archaea (Chistoserdova et al., 1998) and is a coenzyme not abundant in methanogens (Leigh, 1983) but is utilized by most other organisms (Rouviere and Wolfe, 1988). The enzyme 4-(β-D-ribosfuranosyl)aminobenzene 5'-phosphate (**β-RFA-P**) synthase catalyzes the first step in the biosynthesis of H_4MPT (White, 1996). The proposed mechanism of β-RFA-P synthase includes the formation of an oxycarbenium intermediate from 5-phospho-α-D-ribosyl-1-pyrophosphate (**PRPP**) and the activation of

para-aminobenzoic acid (**pABA**) to stimulate a nucleophilic attack on PRPP, resulting in the formation of β-RFA-P from the condensation of the C-4 of pABA with C-1 of the ribose ring of PRPP (Rasche and White, 1998; Dumitru and Ragsdale, 2004) (Figure 1.4). Because the C-4 of pABA serves as a nucleophile, this enzyme's mechanism differs from that of other phosphoribosyltransferases (such as the enzyme that catalyzes the rate-limiting step in H_4F biosynthesis) which utilize NH_2 group of pABA as a nucleophile (Rasche and White, 1998). Based on β-RFA-P synthase's uniqueness to methanogens within the ruminal environment and because of this enzymes distinct mechanism relative to other enzymes that utilize pABA, β-RFA-P synthase is considered a promising target for the specific inhibition of ruminal methanogenesis.

Rasche and White (1998) reported that several para-substituted analogs of pABA inhibted β-RFA-P synthase, wheras analogs that had a non-existent, altered, or replaced carboxylic acid group did not inhibit the enzyme. This work was followed up by Dumitru et al. (2003) who reported a number of N-substituted derivatives of pABA that inhibit β-RFA-P synthase, arrest CH_4 production and growth of pure cultures of methanogens, and do not negatively impact the growth of an acetogenic bacterium. Furthermore, 3 of these reported inhibitors decreased CH_4 production in cultures of ruminal fluid without adversely affecting VFA production. However, the concentration of these compounds required to achieve 97-100% inhibition of CH_4 production ranged from 5 to 9 mM (Dumitru et al., 2003). The liquid volume (L) of the rumen is estimated to be $BW(kg)^{0.57}$ (Owens and Goetsch, 1988). Thus, a 500-kg animal would have a ruminal liquid volume of 35 L. Initial achievement of a 5 mM concentration (assuming a

compound MW of 200 g/mol) would require the administration of 35 g. Assuming a liquid passage rate of 7 %/h, maintenance of a 5 mM concentration would require the continual administration of 59 g/d. This can be compared the 0.36 g/d dosage that monensin is currently labeled to be administered to feedlot cattle (Elanco Circulatory AF0480-50B). Based on these values, an inhibitor of methanogenesis that is to be administered orally must have an inhibitory effect at sub-micromolar concentrations. The rationale for, and initial success found in, inhibiting β-RFA-P synthase provides much potential for the future discovery of other pABA analogs capable of inhibition at lesser concentrations.

Sarsaponin. The plant *Yucca shidigera* can be found growing in the southwestern part of the United States and in Mexico (Cheeke and Shull, 1985). It is grazed by cattle in times of drought and feed shortage (Cheeke and Shull, 1985). The active ingredients found in extracts of *Yucca shidigera* are steroidal saponins (referred to as sarsaponin), which have detergent properties and antifungal activity (Osbourn, 1996). There is much variability in the effects of sarsaponin on ruminal fermentation, but there is clear evidence indicating that sarsaponin does alter the ruminal microbial population. The addition of sarsaponin to the diets of dairy cows has been reported to enhance (Goetsch and Owens, 1985), diminish (Goetsch and Owens, 1985; Valdez et al., 1986), or not affect (Wu et al., 1994) the digestibility of the diets. The response to sarsaponin supplementation may be a function of the diet with the positive responses in digestibility observed with the medium- to low-concentrate diets (Goetsch and Owens, 1985). The idea of sarsaponin's differential effects with different diets is supported by in vitro data

indicating that the negative effects of sarsaponin on digestive microbes is less pronounced with grain digestion compared to forage (Wang et al., 2000b). Results from other experiments evaluating the effects of sarsaponin on ruminal microbe populations indicate no effect (Wang et al., 1998) or an increase (Valdez et al., 1986) in the total bacterial population, and a decrease in protozoal numbers has been consistently observed (Valdez et al., 1986; Wallace et al., 1994; Wang et al., 1998; Hristov et al., 1999; Pen et al., 2006). The addition of sarsaponin to the growth medium of pure cultures differentially affected the growth of several different species of ruminal bacteria (Wallace et al., 1994; Wang et al., 2000a). In general, sarsaponin inhibits cellulolytic ruminal bacteria, but its effect on amylolytic bacteria is species-dependent with gram-positive bacteria more prone to inhibition (Wang et al., 2000a). Because of sarsaponin's effect on the ruminal microbe population, it is not surprising that the products of ruminal fermentation are also often altered following sarsaponin treatment.

In cultures provided a 1:9 (forage:concentrate) substrate, sarsaponin decreased the total VFA concentration when the culture pH was 7.0, but increased the VFA concentration when the culture pH was 5.5 (Cardozo et al., 2005). Therefore the effect of sarsaponin may depend on pH and effects may be more favorable at a lesser pH. The acetate:propionate ratio was decreased by sarsaponin when culture pH was 5.5 (Cardozo et al., 2005) with similar effects on VFA production being reported by other investigators from in vitro (Pen et al., 2006) and in vivo (Hristov et al., 1999; Santoso et al., 2004) experiments. Total gas production was not affected or decreased in cultures treated with sarsaponin and provided barley grain or alflalfa hay, respectively, as a substrate (Wang et

al., 2000b). Furthermore, sarsaponin decreased CH_4 produced by cultures of ruminal fluid (Lila et al., 2003; Pen et al., 2006) and also decreased in vivo CH_4 production (Santoso et al., 2004). Taken together, the data from these studies indicate that the effect of sarsaponin is likely dependent upon diet and pH which are often related. Sarsaponin does inhibit CH_4 production but this treatment's efficacy for chronic inhibition of methanogenesis has yet to be determined.

Feeding Strategies That Reduce Ruminal CH_4 Emissions

Type of carbohydrate. The amount of CH_4 produced during ruminal fermentation is dependent upon the nature of the substrate being fermented. In general, methanogenic potential of the ruminal microflora is greatest for the fermentation of structural carbohydrates compared to that of non-structural carbohydrates (Johnson and Johnson, 1995; Johnson et al., 2000; Boadi et al., 2004). More specifically, CH_4 production per gram of digested cellulose was calculated to be 3 times that per gram of digested hemicellulose and 5 times that per gram of digested soluble residue with the latter consisting of primarily starch (Moe and Tyrrell, 1979).

A modeling approach was used to estimate the methanogenic potential of several different diets (Benchaar et al., 2001), and the pertinent results of this study are summarized in Table 1.2. In general, replacement of forage with concentrate decreases CH_4 production. With equal DM intakes across all treatments the amount of CH_4 produced (Mcal/d) increases as the proportion of concentrate in the diet is increased from 0 to 20%, but CH_4 production decreases as greater than 20% of the diet is composed of concentrate (Benchaar et al., 2001). Similarly, Blaxter and Wainman (1964) reported that

CH_4 production increases as corn replaces hay in the diet for 20 and 40% but declines markedly as the proportion of corn in the diet increases to 60, 85, and 95%, but Orskov et al. (1968) reported a steady decline in CH_4 production as the proportion of concentrate in the diet was increased from 0 to 80% . These results indicate that in general, diets containing a large amount of starch do not favor methanogenesis.

Diets composed of a starchy concentrate (barely; 20% starch and 23% NDF) result in 23.4% less feed GE lost as CH_4 compared to a diet composed of a fibrous concentrate (beet pulp; 2% starch and 31% NDF) (Benchaar et al., 2001). The results of this modeled scenario were supported when the ratio of structural to nonstructural carbohydrates was adjusted to create low- and high-concentrate diets fed to steers, and the high-concentrate diet resulted in a lesser percentage of feed GE lost as CH_4 (Guan et al., 2006). Therefore, replacement of dietary NDF with dietary starch may be an effective strategy for decreasing ruminal methanogenesis.

Aside from replacing structural carbohydrates with non-structural carbohydrates in the diet, adjusting the amount or source of a single type of carbohydrate may affect methanogenesis. When comparing alfalfa of midbloom and vegetative maturities (47 and 31% NDF, respectively) the latter would result in 16% less CH_4 when expressed as a percentage of feed GE (Benchaar et al., 2001). Therefore, simply decreasing the NDF content of a diet may be another feeding strategy for decreasing ruminal methanogenesis. The source of carbohydrate may also affect CH_4 production. Despite similar starch contents, diets composed of corn result in 16% less feed GE lost as CH_4 compared to diets composed of barley (Benchaar et al., 2001). In support of this estimate,

Beauchemin and McGinn (2005) reported that steers consuming a corn-based finishing diet lost 30% less feed GE as CH_4 compared to steers consuming a barley-based diet. This reduction in ruminal CH_4 production may be attributed to the lesser ruminal digestibility of the corn starch compared to the barley starch (Theurer et al., 1987). If the starch escaping the rumen is efficiently digested in the small intestine then altering dietary starch source is an effective strategy for decreasing CH_4 emissions. However, if the starch passes the small intestine and is fermented in the large intestine, then the difference in total CH_4 emissions between the two sources will be decreased (Benchaar et al., 2001).

Dietary fat. The addition of fat or individual fatty acids to ruminal cultures decreases CH_4 production (Dong et al., 1997; Dohme et al., 1999; Dohme et al., 2001; Soliva et al., 2003; Soliva et al., 2004). Also, in vivo CH_4 production is reduced if the fat or fatty acids are added directly to the rumen (Czerkawski et al., 1966) or to ruminant diets (Towne et al., 1990; Machmuller and Kreuzer, 1999; Johnson et al., 2002; Machmuller et al., 2003a; Machmuller et al., 2003b; Mcginn et al., 2004; Jordan et al., 2006a; Jordan et al., 2006b). The mechanism by which fat depresses methanogenesis has not been defined but is likely a combination of biohydrogenation of unsaturated fatty acids and direct inhibition of protozoa, bacteria, and methanogens.

Biohydrogenation of unsaturated fatty acids would decrease the amount of H_2 available for the reduction of CO_2 and this may be one mechanism by which the addition of fat to ruminant diets decreases ruminal methanogenesis. Following infusion into the rumens of sheep, unsaturated fatty acids (oleic acid, 18:1; linoleic acid, 18:2; and

linolenic acid, 18:3) were hydrogenated and CH_4 production was inhibited, with the inhibition being greater when the dosage and unsaturation of the fatty acids increased (Czerkawski et al., 1966). However, the authors cautioned that the decrease in methanogenesis may not be solely attributed to biohydrogenation because: 1) there was not a direct link between the number of moles of double bonds and the depression in CH_4 production and 2) palmitic acid (16:0) acid also induced an inhibition of methanogenesis, but to a lesser extent than the other unsaturated, long-chain fatty acids. Biohydrogenation may be partially responsible for the depression in methanogenesis induced by unsaturated fatty acids but may not explain this reduction in its entirety.

Approximately 20% of ruminal methanogens are associated with protozoa (Stumm et al., 1982), and these methanogens may account for up to 25% of ruminal methanogenesis (Newbold et al., 1995). Therefore, in theory, treatments that adversely affect protozoal populations will inhibit methanogenesis. However, fatty acids seem to have a direct inhibitory effect on methanogens. Coconut oil, a lipid rich in medium-chain fatty acids, decreases the number of protozoa in faunated ruminal cultures, suppresses CH_4 production in both faunated and defaunated cultures to a similar extent, and reduces the population of methanogens in cultures independent of the status of protozoa (Dohme et al., 1999). These results indicate that medium-chain fatty acids may suppress methanogenesis by independently acting on both protozoa and methanogens. This is supported by the report that myristic acid (14:0) does not affect the population of protozoa in vitro but does suppress in vitro methanogenesis more than caprylic acid (8:0) or capric acid (10:0) which both decrease the protozoal count (Dohme et al., 2001).

Therefore, the methanogenic suppressing and anti-protozoal actions of medium-chain fatty acids my act synergistically to inhibit ruminal methanogenesis.

Evaluation of the effects of several fatty acids (8:0, 10:0, 12:0, 14:0, 16:0, 18:0, and 18:2) revealed that lauric acid, myristic acid, and linoleic acid were the most potent suppressors of in vitro methanogenesis (Dohme et al., 2001). Coconut oil, which contains predominantly lauric acid (12:0) and myristic acid, inhibits in vitro (Dong et al., 1997; Dohme et al., 1999) and in vivo (Machmuller and Kreuzer, 1999; Machmuller et al., 2003a; Jordan et al., 2006b) methanogenesis. In vivo CH_4 production is also suppressed when sheep are administered myristic acid (Machmuller et al., 2003b), or oleic, linoleic, or linolenic acid (Czerkawski et al., 1966). Therefore, increasing the dietary proportion of medium- or long-chain fatty acids may be yet another feeding strategy for reducing ruminal methanogenesis.

Coproducts of the Corn-Ethanol Industry

Feeding DDGS and WDGS. Recent demand for ethanol is strong and this demand is driven largely by the Clean Air Act amendment of 1990, which requires the use of reformulated gasoline to reduce air pollutants (Rausch and Belyea, 2006b). Ethanol can be produced from corn by the dry grind process, which results in wet distillers grains plus solubles (WDGS) or dried distillers grains plus solubles (DDGS) as coproducts (Stock et al., 2000). In the past five years the production of distillers grains has increased from 2.3 to 9.0 million metric tons, and 75 to 80% of this is fed to ruminant animals (RFA, 2005). Increased acceptance and usage of this ethanol production coproduct as a feed source stimulated research focused on determining what dietary

components may be replaced with distillers grains and at what level they may be fed to optimize animal performance. Stock et al. (2000) reviewed trials conducted from 1980 to 1985. They concluded that distiller grains has an average energy value of 109% that of corn, and this estimate included inclusion rates of distillers grains ranging from 10 to 65% of the diet DM. Several investigators have focused on replacing corn in feedlot diets, and found that WDGS improves average daily gain (Larson et al., 1993; Ham et al., 1994; Lodge et al., 1997; Trenkle, 1997a; Trenkle, 1997b; Al-Suwaiegh et al., 2002; Vander Pol et al., 2006; Buckner et al., 2007; Corrigan et al., 2007) and that the energy value is optimized at inclusion levels ranging from 20% to 40% (Larson et al., 1993; Trenkle, 1997a; Trenkle, 1997b; Vander Pol et al., 2006).

The reason the energy value of WDGS and DDGS exceed that of corn in not known. Stock et al. (2000) proposed that increased energy value may be attributed to a lesser incidence of subacute acidosis, improved energy utilization, or a greater fat content. The increased energy content of these feedstuffs may be a direct result of the latter as WDGS and DDGS do contain a greater amount of energy-rich fat when compared to corn (4% vs 10%) (NRC, 2000). However, the increased fat content may also be indirectly increasing the energy value of WDGS and DDGS as the fatty acids present (USDA ARS, 2005) have been reported to specifically inhibit methanogenesis (Dohme et al., 2001). Therefore, the improved energy value of these coproducts of the ethanol industry may be due to a decreased fractional loss of feed GE to ruminal CH_4 production, but the effect of dietary WDGS or DDGS on ruminal CH_4 production has not yet been examined.

Conclusion

Ruminal methanogenesis is advantageous to the host but attenuation of this process will theoretically increase the efficiency of ruminal fermentation. Dietary strategies that lessen the amount of CH_4 produced per unit of digested DM include the replacement of structural carbohydrates with non-structural carbohydrates, and increasing the fat content of diets. Specific inhibition of methanogenesis with anti-methanogenic compounds (such as BES) have proven unsuccessful because methanogens quickly become resistant to the effects of treatment. Data from experiments examining the effects of other compounds (analogs of pABA and sarsaponin) indicate these treatments to be potent inhibitors of methanogenesis, but their ability to inhibit CH_4 production following chronic exposure has yet to be examined. Because the majority of ruminal methanogens are difficult to culture, the effects of dietary or anti-methanogenic treatments on these microorganisms have not been extensively studied. Molecular techniques revealed the true diversity of the ruminal methanogen population and are beginning to be used to characterize changes in this population following treatments. Understanding the differential changes that take place following the imposition of treatments will expedite the process of identifying a treatment or combination of treatments that will successfully inhibit ruminal CH_4 emissions following chronic exposure.

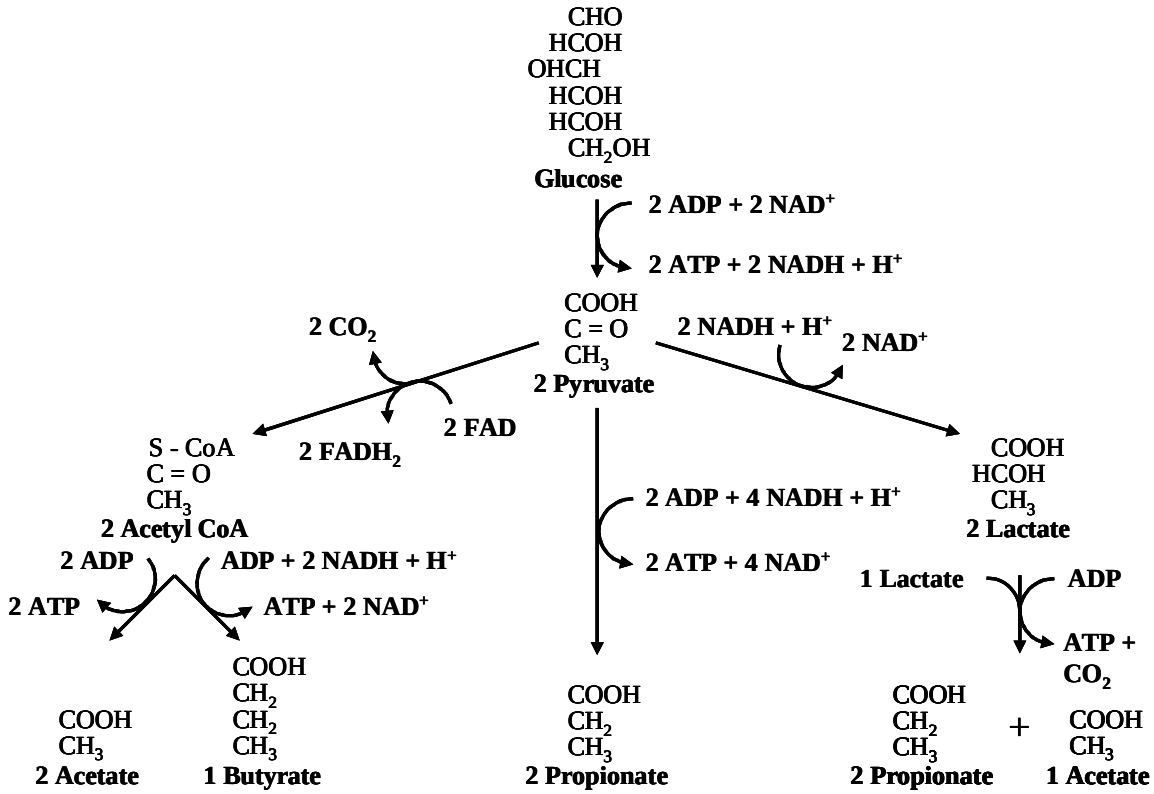
Table 1.1. Anthropogenic sources of greenhouse gases in the United States (Tg CO_2 equivalents). Adapted from EPA (2007).

	Year			
	1990	1995	2000	2005
CO_2	**5,062**	**5,385**	**5,940**	**6,090**
CH_4	**609[a]**	**599[a]**	**564[a]**	**539[a]**
Landfills	161	157	132	132
Enteric fermentation	116[a]	121[a]	114[a]	112[a]
Beef cattle	*81*	*87*	*81*	*79*
Dairy cattle	*29*	*28*	*27*	*28*
Horses	*1.9*	*1.9*	*2.0*	*2.0*
Sheep	*1.9*	*1.5*	*1.2*	*1.0*
Swine	*1.7*	*1.9*	*1.9*	*1.9*
Goats	*0.3*	*0.2*	*0.3*	*0.3*
Manure Management	31	35	39	41
Other	301	286	279	254
N_2O	**482**	**484**	**500**	**469**
HFC's, PFC's and SF_6	**89**	**104**	**144**	**163**
Total	**6,242[a]**	**6,571[a]**	**7,147[a]**	**7,260[a]**

[a] Totals may not sum due to rounding of individual numbers.

Table 1.2. Effect of different diets on ruminal methane (CH_4) production (Benchaar et al., 2001).

	NDF (% DM)	Starch (% DM)	CH_4 (Mcal/d)	CH_4 (% GE Intake)
Forage/concentrate Ratio				
100 / 0	42	-	2.55	4.98
80 / 20	37	7	2.70	4.83
50 / 50	27	17	2.61	4.43
30 / 70	20	24	2.12	3.46
Fibrous vs. Starchy Concentrate				
Fibrous: beet pulp	31	2	2.72	5.07
Starchy: barley	23	20	2.34	3.86
Alfalfa Hay Maturity				
Midbloom	47	-	1.73	3.17
Vegetative	31	-	1.80	3.64
Rapidly vs. Slowly Degraded Starch				
Barley	28	42	2.94	4.63
Corn	22	50	2.53	3.91



Net Yield Per 1 Molecule of Glucose

ATP	**4**	**3**	**4**	**2**
Reduced Cofactors	**4**	**2**	**-2**	**0**

Figure 1.1. Schematic representation of the dominant pathways associated with VFA production in the rumen. Adapted from Herdt, 2002.

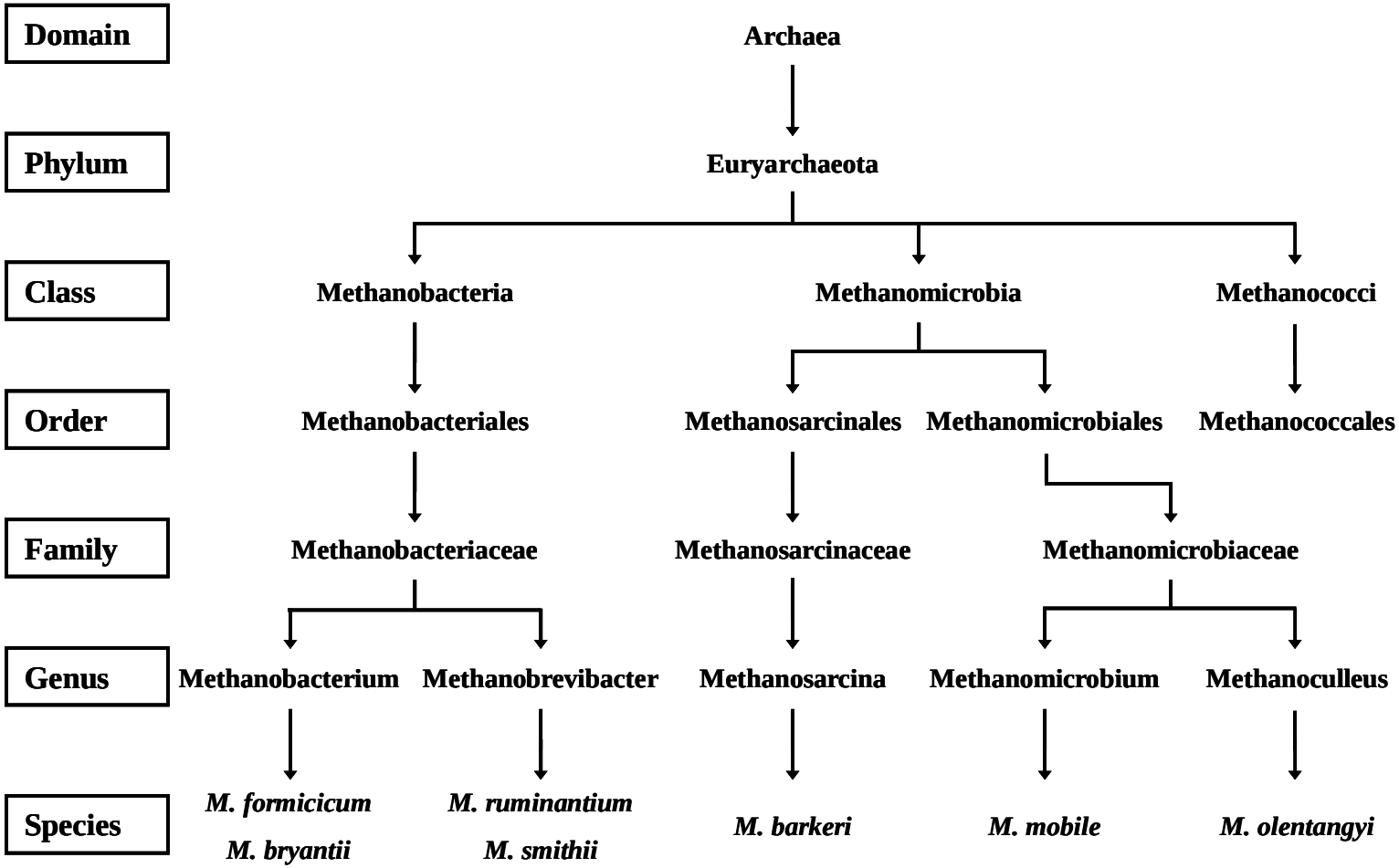


Figure 1.2. Lineage of the four orders of methanogenic Archaea identified by DNA hybridization and the seven species isolated from samples of ruminal contents.

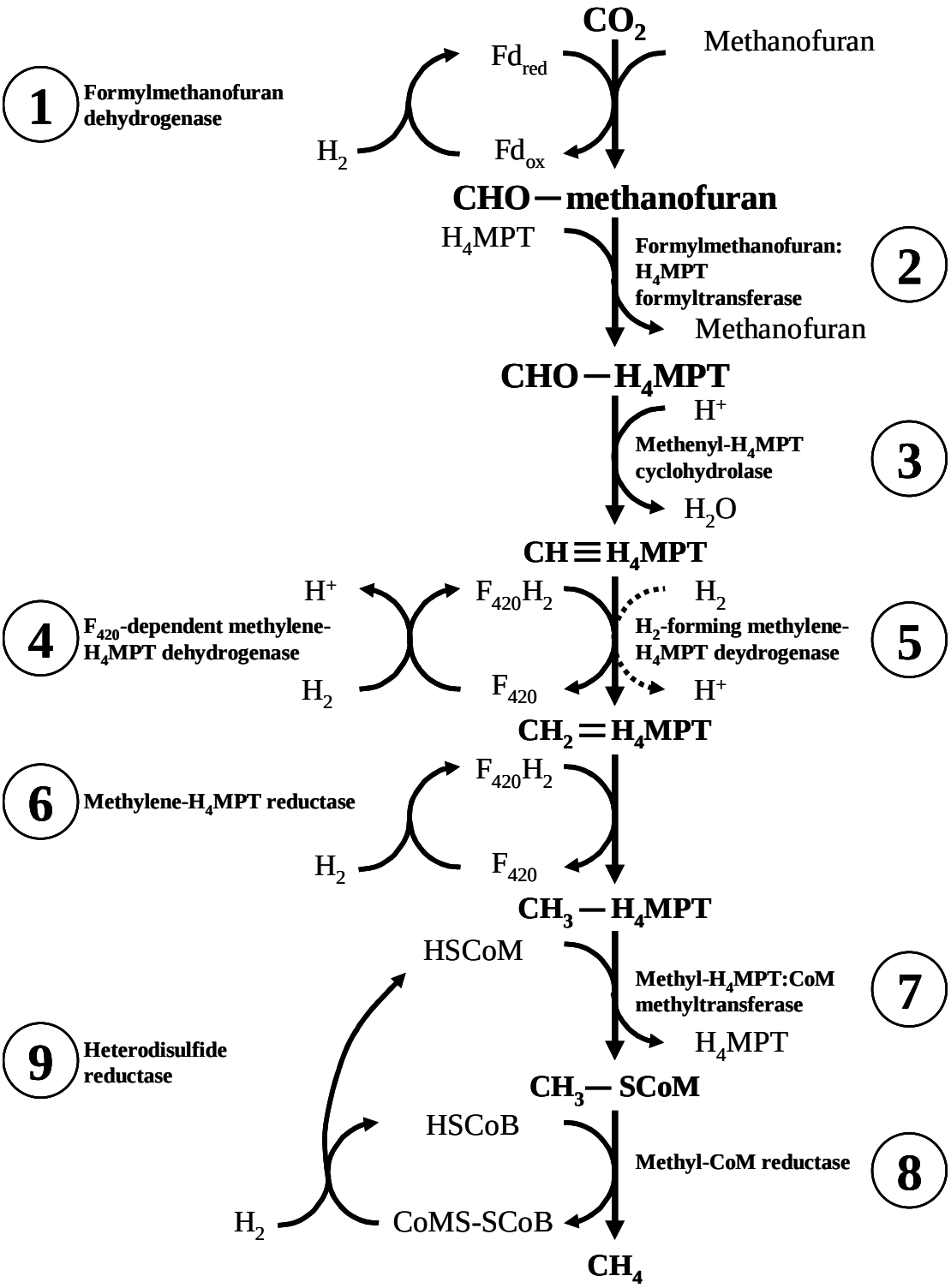


Figure 1.3. Production of (methane) CH_4 from the stepwise reduction of CO_2 with H_2. Adapted from (Ferry and Kastead, 2007).

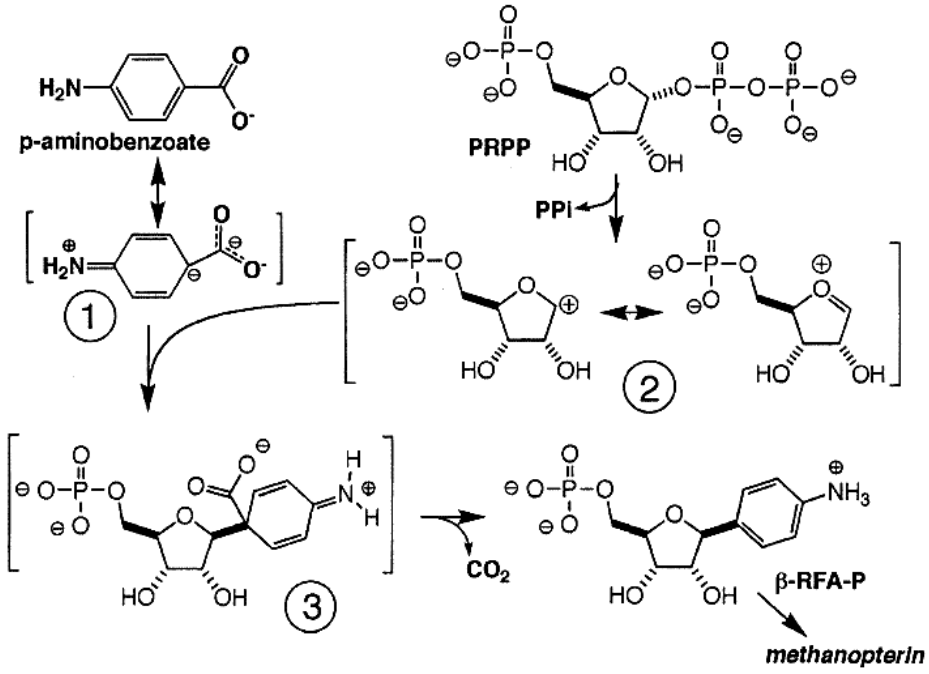


Figure 1.4. Proposed mechanism for the enzyme 4-(β-D-ribosfuranosyl)aminobenzene 5'-phosphate, which catalyzes the first committed step in the production of the cofactor tetrahydromethanopterin (Dumitru, 2005).

CHAPTER II

Chronic exposure of ruminal fluid cultures to treatments that inhibit methanogenesis

ABSTRACT: Ruminal methanogens adapt to treatments such as 2-bromoethanesulfonate (BES) that inhibit methanogenesis. Acute treatment of ruminal cultures with a nitrofuranyl derivative of para-aminobenzoate (NFP) and an extract from the plant *Yucca shidigera* (Yucca) inhibit methane (CH_4) production. Our objective was to determine the extent to which ruminal cultures acquire resistance following chronic exposure to NFP, Yucca, and BES. Eight cultures (n = 2 per treatment) were inoculated with ruminal fluid and chronically exposed in duplicate to: 1) control; 2) 100 μM NFP; 3) 10 μM BES; or 4) 2.5 μL/mL Yucca. Every two days 50% of each culture was replaced with fresh medium. On d 2, 10, 22, 32, 40, 60, and 90, chronic cultures were used to inoculate acute cultures. Acute cultures were treated in duplicate with either 0 or 10X the same inhibitor as used for creating the chronic culture inoculum. Control-inoculated acute cultures were also treated in duplicate as controls and with a 10X concentration of NFP, Yucca, and BES. The 10X concentration of NFP, Yucca, and BES decreased ($P < 0.01$) CH_4 production in control-inoculated cultures. Resistance developed to the inhibitory effects of BES and Yucca but not to NFP. Methanomicrobiales and Methanobacteriales increased in abundance following chronic BES and Yuccca treatments, respectively. The abundance of Bacteria was not decreased by NFP, Yucca, or BES. We conclude that ruminal methanogens acquire resistance to the effects of Yucca following chronic exposure but remain sensitive to the inhibitory actions of NFP for at least 90 d.

INTRODUCTION

Ruminal methanogens consume carbon dioxide and hydrogen thereby diminishing the utility of ruminal digestion by depleting substrates that could instead be used by bacteria to make VFA. Estimates for feed GE lost due to ruminal methane (**CH_4**) production range from 2 to 12% (Johnson and Johnson, 1995). Methane is a potent greenhouse gas, and anthropogenic sources are responsible for 55-70% of total CH_4 emissions (IPCC, 2001). Enteric fermentation is responsible for 20% of total CH_4 emissions from anthropogenic activities, with beef and dairy cattle being the largest emitters of CH_4 (EPA, 2007). Inhibition of ruminal CH_4 production may improve the efficiency of ruminal fermentation while decreasing the atmospheric concentration of a greenhouse gas.

The compound 2-bromoethanesulfonate (**BES**) is structural analog of conenzyme M. It can block the reductive demethylation of CH_3-S-CoM in the last step of methanogenesis (Balch and Wolfe, 1979b). Selective inhibition of ruminal methanogens following BES treatment has been reported in vitro (Martin and Macy, 1985; Immig et al., 1996; Nollet et al., 1997; Ungerfeld et al., 2004) and in vivo (Immig et al., 1996), but ruminal methanogens acquire resistance to BES treatment after 3 d of continuous treatment in vivo and CH_4 production resumes (Immig et al., 1996).

A chronically effective methanogenesis inhibitor is desired. The enzyme 4-(β-D-ribosfuranosyl)aminobenzene 5'-phosphate (**β-RFA-P**) synthase catalyzes the first step in the biosynthesis of tetrahydromethanopterin (**H_4MPT**)(Dumitru and Ragsdale, 2004), which is a cofactor essential to methanogenesis and unique to Archaea in the ruminal

environment. Specific analogs of *para*-aminobenzoate (**pABA**), a substrate for β-RFA-P synthase, can arrest both methanogenesis and growth of methanogens yet not adversely affect the growth of an acetogen (Dumitru et al., 2003). A nitrofuranyl derivative of pABA (**NFP**; Figure 2.1) inhibits methanogenesis in 22-h ruminal cultures by 99, 99, and 19% at concentrations of 5.0, 1.0 and 0.1 mM, respectively (Behlke et al., 2005). An extract from the plant *Yucca shidigera* (**Yucca**) is a product commercially available to livestock producers and contains a group of steroidal glycosides collectively called sarsaponin. Sarsaponin possesses anti-protozoal properties (Wallace et al., 1994; Hristov et al., 1999) and suppressed CH_4 production by > 50% (Lila et al., 2003). Methanogens derive energy for growth from the process of methanogenesis (Ferry and Kastead, 2007). Anti-methanogenic compounds should inhibit the growth of at least some ruminal methanogens.

The objectives of this work were to determine: 1) The extent to which ruminal cultures acquire resistance following chronic exposure to NFP, Yucca, and BES (BES served as a positive control for resistance development), and 2) if a relationship exists between in vitro CH_4 production and ribosomal DNA indicators of methanogens.

MATERIALS AND METHODS

Ruminal Sampling and Animal Care. The ruminal fluid used as the initial inocula source was collected from a fistulated heifer allowed a mixed forage and concentrate diet (Table 2.1). Ruminal fluid was collected through a rumen cannula using a suction strainer, strained through four layers of cheesecloth, and immediately

transported to the laboratory. The procedures used were approved by the University of Nebraska's Institutional Animal Care and Use Committee (protocol #04-05-032).

Inhibitors. BES was purchased from Sigma-Aldrich. The nitrofuranyl derivative of pABA (Figure 2.1) was synthesized by PharmAgra Labs, Inc. (Brevard, North Carolina). Yucca was provided by SarTec Corporation (Anoka, Minnesota).

Chronic Cultures. Ruminal fluid was used to inoculate chronic cultures (n = 2 per treatment) exposed to no treatment (control) or a 1X concentration of NFP, Yucca, or BES (100 µM, 2.5 µL/mL, and 10 µM, respectively). In addition to 4 mL of ruminal fluid, the chronic cultures contained 8 mL of McDougall's buffer ((McDougall, 1948); 0.1 M $NaHCO_3$, 0.02 mM Na_2HPO_4, 8 mM KCl, 8 mM NaCl, 0.5 mM $MgSO_4 \cdot 7H_20$, and 1 mM $CaCl_2 \cdot 2H_20$), 8 mL of distilled H_2O, 40 mg of cellobiose, 40 mg trypticase, 20 µg of resazurin, 25 µL of a micro mineral solution (0.5 M $MnCl_2 \cdot 4H_20$, .04 M $CoCl_2 \cdot 6H_2O$, and 0.3 M $FeCl_3 \cdot 6H_2O$), and 20 mg of Na_2S. The fermentation media were gassed with CO_2 to create oxygen-free media and then added to 120 mL glass vials which contained the respective treatments while oxygen free gas (H_2/CO_2, 80:20) was projected into each vial. The vials were sealed, pressurized to 100 kPa above atmospheric pressure, and allowed to incubate in a water bath (39°C) for 90 days. Every 2 days 50% of the media from each vial were replaced with fresh culture media which contained the same components as the original media except that clarified ruminal fluid (supernatant collected from 2 centrifugation steps of 12,000 x g for 30 min) was substituted for fresh ruminal fluid. The fresh medium contained the same treatment component as the

medium it replaced thus allowing for the concentration of inhibitor to remain constant for the duration of the experiment.

Acute Cultures. Excluding inocula source, the acute cultures contained proportionally identical ingredients compared to the chronic cultures and were prepared identically as 4 mL of fermentation media in 10 mL glass vials incubated in a water bath (39ºC) for 18 h. On d 0, ruminal fluid from the same source as that used to inoculate chronic cultures was used to inoculate acute cultures (n = 4 per treatment) that received no treatment (control), NFP (100 µM or 1,000 µM), Yucca (2.5 µL/mL or 25 µL/mL) or BES (10 µM or 100 µM). On d 2, 10, 22, 32, 40, 60, and 90, media removed from chronic cultures were used to inoculate acute cultures that were treated in duplicate with either 0 or 10X the same inhibitor as used for creating the chronic culture inoculum (n = 24 per day). Control-inoculated acute cultures were also treated in duplicate as controls and with these 10X doses of NFP, BES, and Yucca (n = 16 per day). The media remaining after inoculation of acute cultures were frozen (-20ºC) and genomic DNA was later extracted.

CH_4 Analysis. Following incubation, acute cultures were cooled to room temperature and the headspace pressure was measured using a micromanometer. Methane concentration was assayed by gas chromatography using a packed column (Alltech; silica gel 60/80 grade 12, 18’ x 1/8” x .085” SS) and thermal conductivity detector. Injector, detector, and column temperatures were 120, 120, and 60°C, respectively.

DNA Extraction and Real-time PCR. Total genomic DNA was extracted from media removed from chronic cultures utilizing the RBB+C method (Yu and Morrison, 2004), which employs a QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, California) following repeated bead beating steps to disrupt cell membranes. The quality and quantity of DNA samples were determined with gel electrophoresis and by spectrophotometry, respectively. The relative presence of targets for 2 domain-specific (Bacteria and Archaea) and 4 order-specific (Methanobacteriales, Methanomicrobiales, Methanococcales, and Methanosarcinales) primer and probe sets (Yu et al., 2005) was determined for each sample in duplicate with real-time PCR utilizing an AB7700 (Applied Biosystems, Foster City, California). Each reaction well contained 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 4.2 µL of community DNA, 1.25 µL of the forward and reverse primers (final concentration, 500 nM), 0.4 µL of the corresponding probe (final concentration, 150 nM), and nuclease-free water to a final volume of 25 µL. The following reaction conditions were applied to each well: an initial 2-min incubation at 50ºC; a 10-min incubation at 95ºC; and 45 cycles of denaturation at 95ºC for 10 s, annealing at 55ºC for 30 s, and extending at 60ºC for 1 min.

An optimal in-well DNA concentration was established for each primer and probe set to maximize the DNA present in each well and allow for the detection of targets present in small amounts while avoiding the inhibitory effects associated with most DNA samples (Gallup and Ackermann, 2006). The threshold cycle (**Ct**) values for each primer and probe set were determined with a test plate containing serially diluted samples from a pooled stock. This stock was created by combining aliquots of each experimental

sample. The Ct values were then plotted against the $LOG_{(10)}$ of the dilution factor, and the linear portion of this plot indicated the concentrations of DNA at which inhibition was absent. The DNA concentration used for each primer and probe set was 60% less than the maximum determined to avoid inhibition of the PCR reaction. The optimal in-well DNA concentrations for the primer and probe sets targeting Archaea, Methanobacteriales, Methanomicrobiales, and Bacteria were 0.1381, 0.0552, 0.0228, and 0.1381 ng/µL, respectively. Four samples containing decreasing concentrations of DNA and diluted from the stock solution were tested in duplicate on each PCR plate. The difference between the average Ct value of the standards on all plates and the average Ct value for the 4 standards on each plate was added to the Ct of all samples on each respective plate to neutralize inter-plate variation. The Ct of each plate's four standards was plotted on the $LOG_{(10)}$ of the dilution factor. The slope of this line indicated the efficiency of amplification (**E_{AMP}**). The Ct value for each unknown was then standardized to a common denominator with the equation:

$$Ct - (LOG_{(E_{AMP})}(\text{Dilution factor})) = \text{Ct/mL of ruminal fluid} \qquad [2.1]$$

For each primer and probe set the average Ct for the control cultures on d 0 was used as a "Reference Ct" and a value of 1.0 relative abundance units was assigned to these samples. The abundance of target for each primer and probe set for all other samples was calculated with the equation:

$$\text{Relative abundance} = \text{Efficiency}^{\Delta Ct(\text{Reference} - \text{Sample})} \qquad [2.2]$$

Statistical Analysis. Data were analyzed utilizing the MIXED procedure of SAS (SAS Inst., Inc., Cary, North Carolina); correct degrees of freedom were obtained using

the KENWARDROGER option. The individual acute culture was the experimental unit for determining the CH_4 production on each day, whereas the individual chronic culture was the experimental unit for relative microorganism quantification. The model for CH_4 produced by d 0 cultures included the fixed effect of treatment. The model for CH_4 produced by d 2, 10, 22, 32, 40, 60 and 90 acute cultures included the fixed effects of previous exposure to a 1X dose of an inhibitor, treatment with a 10X dose of an inhibitor, the random effect of day that the media were removed from continuous cultures and used to inoculate an acute culture, and all appropriate 2-way and 3-way interactions. The model for real-time PCR data included the fixed effect of treatment, the random effect of day, and the treatment by day interaction. All of the real-time PCR data were $LOG_{(10)}$ transformed prior to analysis to account for non-constant variances as indicated by the Shapiro-Wilk test for normality. Because the same medium was sampled across days a repeated measures covariance structure was used. The CS covariance structure was selected based on the Akaike information criterion. An amount of CH_4 that was less than the sensitivity of our gas chromatograph was produced by acute cultures: inoculated with control media and treated with a 10X concentration of either NFP or BES; and inoculated with media previously exposed to NFP and treated with a 10X concentration of NFP. Therefore, these CH_4 production data were excluded from the analysis. Least squares means are reported and were separated using the protected least significant difference test when a significant *F*-test ($P < 0.05$) was detected.

RESULTS AND DISCUSSION

2-bromoethanesulfonate served as a positive control for resistance development in our assay system. 2-bromoethanesulfonate inhibited methanogenesis when included in day 0 acute cultures (Table 2.2), which paralleled previously reported results of in vitro experiments (Martin and Macy, 1985; Immig et al., 1996; Nollet et al., 1997; Ungerfeld et al., 2004). Continuous exposure of ruminal cultures to BES for 2 d created a less methanogenic inoculum source (Figure 2.2). Following 10 d of exposure the inoculum's methanogenic potential in acute cultures was regained. These results indicate that the methanogens present in the continuous culture acquired resistance to the effects of BES in less than 10 d. This is similar to what was observed in vitro (Immig et al., 1996; Ungerfeld et al., 2004) and in vivo (Ungerfeld et al., 2004). These results also validated the efficacy of our culture system for allowing the development of resistance. Despite being resistant to 1X BES, inocula from the chronically exposed BES cultures retained significant sensitivity to 10X BES.

Our primary objective was to determine the extent to which ruminal cultures acquire resistance following chronic exposure to NFP and Yucca. Data from the d 0 acute culture confirmed both that the initial inoculum was methanogenic and that our inhibitors were effective (Table 2.2). The inhibitor concentrations were selected such that the 1X concentration would inhibit CH_4 production by >50% and the 10X concentration near 100%. This was based on a pilot experiment (data not shown). Similar to previous observations (Behlke et al., 2005), treatment of d 0 acute cultures with a 1X (100 µM) and 10X (1,000 µM) concentration of NFP resulted in a 59 and 98%

inhibition of CH_4 production, respectively. Chronic treatment of ruminal cultures with a 1X dose of NFP for 90 d resulted in an inoculum source that remained sensitive to the effects of this inhibitor (Figure 2.3). NFP treatment resulted in an inoculum source that maintained a diminished capacity to support methanogenesis and also remained sensitive to a 10X concentration of NFP. Yucca treatment did reduce the amount of CH_4 produced by acute cultures on day 0, 10, 22, 32, 40, 60 and 90 (Figure 2.4), which supports previous findings (Lila et al., 2003; Pen et al., 2006). Because of the consistent manner in which Yucca inhibited CH_4 production, the inability of Yucca treatment to inhibit methanogenesis in day 2 acute cultures inoculated with control and exposed media is thought by the authors to be an anomaly. In as little as 2 d of Yucca exposure, the inoculum derived from ruminal cultures had acquired resistance to the effects of the lower dose of this treatment. The response of acute cultures inoculated with chronic control cultures to a 10X concentration of Yucca on day 10 confirmed that the Yucca treatment remained effective.

Our secondary objective was to determine if a relationship exists between in vitro CH_4 production and ribosomal DNA indicators of methanogens. Chronic culturing of control media increased the prevalence of total Archaea (Figure 2.5) and Methanomicrobiales (Figure 2.6) but not Methanobacteriales (Figure 2.7). This increase may be attributed to compensation for dilution as ruminal fluid was diluted with McDougall's buffer and water (1:2:2) prior to inoculating cultures. Relative to control cultures a nearly parallel increase in total Archaea and Methanomicrobiales was observed in cultures treated with BES but not those treated with NFP or Yucca. However, also in

relation to control cultures, the relative abundance of Methanobacteriales was greater in cultures treated with Yucca on all days and also those treated with NFP on day 40, 60, and 90.

The relative abundance of total Archaea and Methanomicrobiales in inocula correlate closely with those inocula's potential to generate CH_4 with respect to the observations that NFP changed both CH_4 production and these two measures of the methanogen population. 2-bromoethanesulfonate treatment initially (d 2) decreased CH_4 production in acute cultures and also decreased the population of all measures of methanogens. When the ability of BES treated cultures to produce CH_4 was regained (day 10) so was the relative abundance of total Archaea and both orders of Archaea. On the other hand, Yucca treatment failed to chronically inhibit CH_4 production but did decrease the relative abundance of total Archaea and those of the order Methanomicrobiales. The relative abundance of methanogens of the order Methanobacteriales was increased by Yucca treatment. Taken together, these results indicate an association between methanogenesis and the prevalence of Methanomicrobiales.

Methanogens from four of the five orders have been identified in the ruminal environment. Methanogens of the order Methanobacteriales are often reported to be the most prevalent in the ruminal environment (Sharp et al., 1998; Tokura et al., 1999; Skillman et al., 2006; Nicholson et al., 2007; Wright et al., 2007). Also, methanogens of the order Methanomicrobiales have been reported to be the most (Yanagita et al., 2000; Tajima et al., 2001) or second most (Sharp et al., 1998; Wright et al., 2007) prevalent in

the ruminal environment. There have also been observations of methanogens of the orders Methanosarcinales (Lin et al., 1997; Jarvis et al., 2000; Nicholson et al., 2007; Wright et al., 2007) and Methanococcales (Lin et al., 1997) present in the rumen, but the abundance of these methanogens was much less than that of methanogens of the orders Methanobacteriales and Methanomicrobiales. After validating our primer and probe sets for Methanosarcinales and Methanococcales with genomic DNA and also confirming the size of PCR product (Yu et al., 2005) from our real-time PCR assays with gel electrophoresis, we were unable to detect the presence of methanogens from these two orders. We conclude that either: 1) methanogens of these two orders were not present or 2) their presence was less than the detection limit of our assay. The theoretical detection limits of our assay were 0.13 and 0.23 pg of DNA per uL of culture fluid for the Methanosarcinales and Methanococcales primer and probe sets, respectively.

Compared to control cultures, the relative abundance of Bacteria was not decreased by any treatment (Figure 8). The anti-methanogenic compound NFP was designed to specifically inhibit β-RFA-P synthase, which is responsible for the production of H_4MPT, a cofactor required for methanogenesis (Rouviere and Wolfe, 1988; Graham and White, 2002). Tetrahydromethanopterin has been discovered in one non-ruminal species of bacteria (Chistoserdova et al., 1998) but within the ruminal environment is thought to be unique to methanogens. Other compounds designed to inhibit β-RFA-P synthase have no effect on the growth of pure cultures of an acetogenic bacterium and also do not decrease the production of VFA by ruminal cultures (Dumitru et al., 2003). Data from the present experiment indicate that, similar to other β-RFA-P

synthase inhibitors, NFP does not inhibit the growth of ruminal bacteria and likely indirectly promotes bacterial proliferation. The active ingredients in the Yucca treatment are steroidal saponins (referred to as sarsaponin), which possess detergent properties and antifungal activity (Osbourn, 1996). Data from studies evaluating the effects of sarsaponin on mixed ruminal bacterial populations indicate no effect (Wang et al., 1998) or an increase (Valdez et al., 1986) in the total bacterial population. The addition of *Yucca shidigera* extract to the growth medium of pure cultures differentially affected the growth of several species of ruminal bacteria (Wallace et al., 1994; Wang et al., 2000a). In general, sarsaponin from *Yucca shidigera* extract inhibits cellulolytic ruminal bacteria, but their effects on amylolytic bacteria are species dependent with gram-positive bacteria being more prone to inhibition (Wang et al., 2000a). Data from the present study are in agreement Valdez et al. (1986) in that Yucca increased the relative abundance of bacteria. However, the species of bacteria that flourished were not determined.

To our knowledge, NFP is the only compound that specifically inhibits methanogenesis and maintains its potency during long-term treatment. Consequently, NFP may be efficacious for inhibiting methanogenesis in ruminant animals as it results in a sustained suppression of ruminal methanogens and does not adversely affect ruminal bacteria.

Table 2.1. Composition of diet composition offered to ruminal fluid donor heifer (DM basis).

Ingredient	%
Brome hay	70.13
Dry rolled corn	14.76
Soybean meal	14.76
Sodium chloride	0.30
Trace mineral premix[1]	0.05
Vitamin A, D, and E premix[2]	0.01

[1]Premix contained 6% Zn, 5% Fe, 4% Mn, 2% Cu, 2,000 ppm I, 500 ppm Co.

[2]Premix contained 30,000 IU vitamin A, 6,000 IU vitamin D, 7.5 IU vitamin E per g.

Table 2.2. Least squares means for methane (CH_4) produced by d 0 acute cultures receiving no treatment (control), or a 1X or 10X concentration of NFP, Yucca, or BES.

Treatment	CH_4 (µmol)[a]
Control	110.0
NFP (100 µM)	45.0[b]
NFP (1,000 µM)	1.8[bc]
Yucca (2.5 µL/mL)	69.4[b]
Yucca (25.0 µL/mL)	39.0[bc]
BES (10 µM)	23.3[b]
BES (100 µM)	7.6[bc]

[a]Methane produced by 22 hr cultures. SE = 1.6 µmol.

[b]Differs from control ($P < 0.01$).

[c]Differs from 1X concentration ($P < 0.01$).

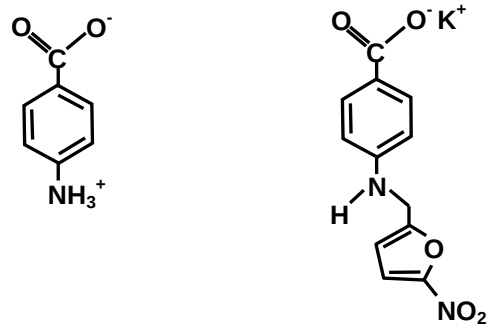


Figure 2.1. Structure of *para*-aminobenzoate (pABA; left) compared to the structure of the nitrofuranyl derivative (NFP; right) of pABA used for this experiment.

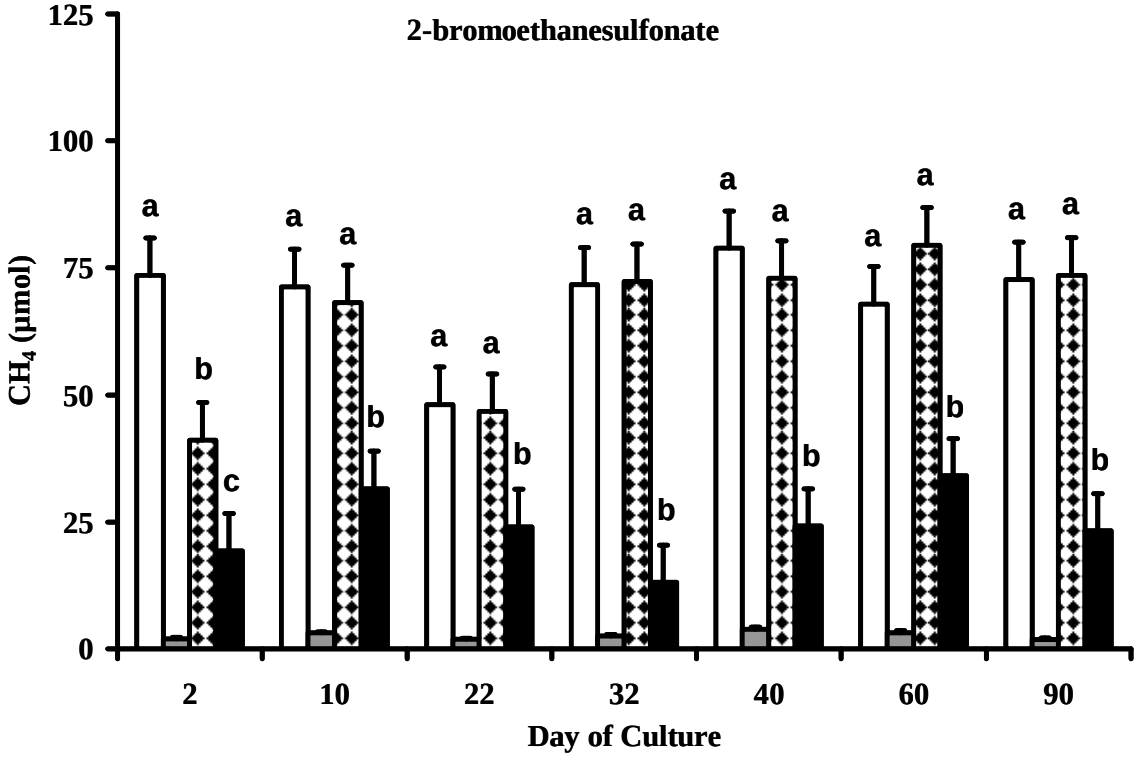


Figure 2.2. Least squares means ± SEM for methane (CH_4) produced by acute cultures inoculated with media from chronic control cultures and receiving no treatment (open bars) or a 10X concentration of 2-bromoethansulfonate (BES; gray bars), or inoculated with media from cultures chronically treated with BES and receiving no treatment (checkered bars) or a 10X concentration of BES (black bars). [a, b, c] **Least squares means with different superscript within day differ ($P < 0.05$).**

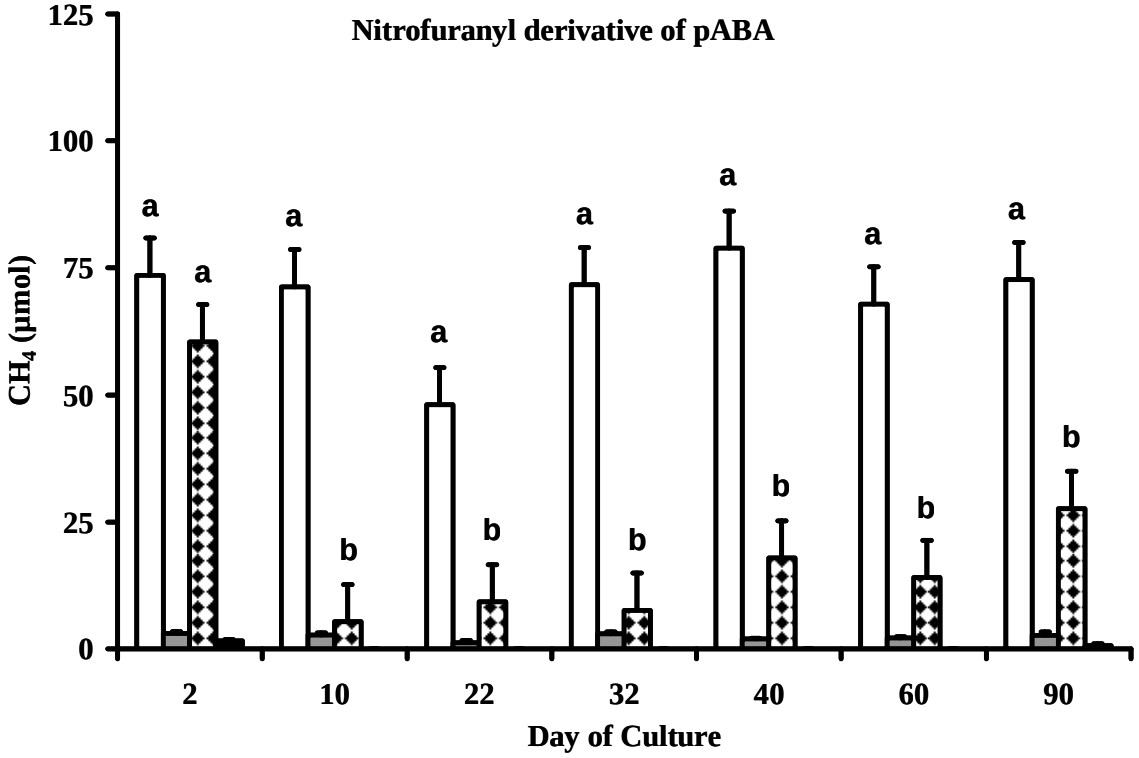


Figure 2.3. Least squares means ± SEM for methane (CH_4) produced by acute cultures inoculated with media from chronic control cultures and receiving no treatment (open bars) or a 10X concentration of a nitrofuranyl derivative of para-aminobenzoate (NFP; gray bars), or inoculated with media from cultures chronically treated with NFP and receiving no treatment (checkered bars) or a 10X concentration of NFP (black bars). [a, b] Least squares means with different superscript within day differ ($P < 0.05$).

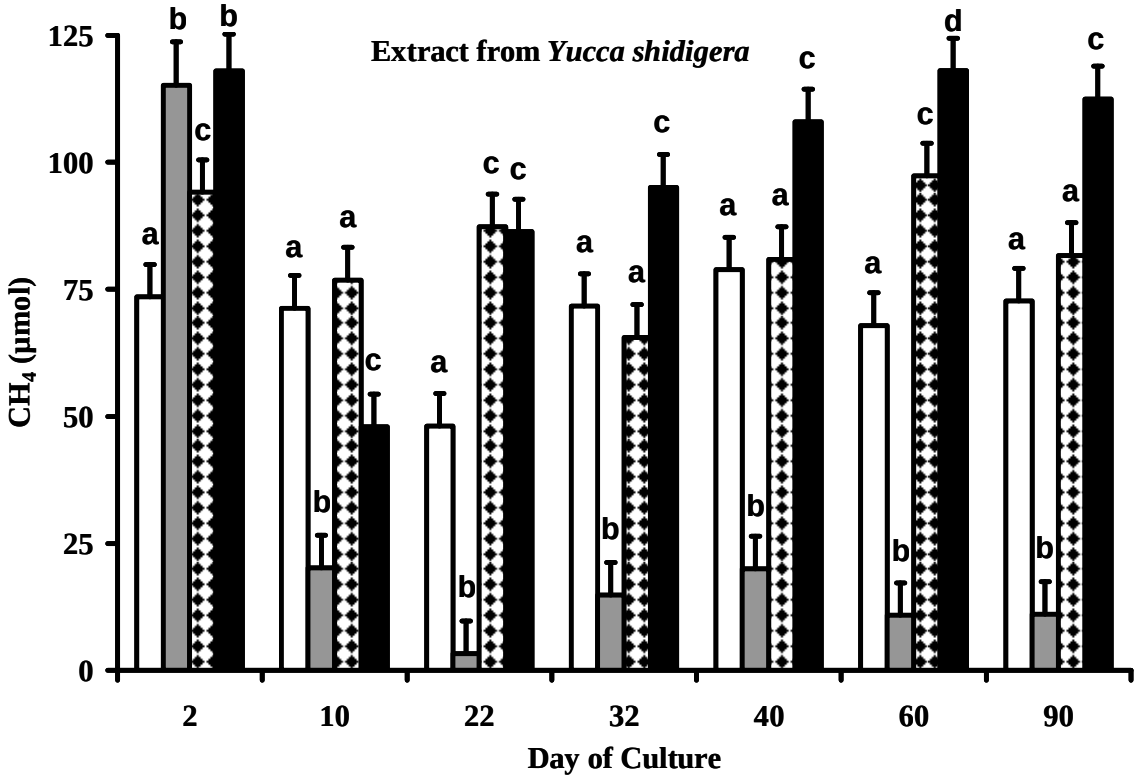


Figure 2.4. Least squares means ± SEM for methane (CH_4) produced by acute cultures inoculated with media from chronic control cultures and receiving no treatment (open bars) or a 10X concentration of an extract from the plant *Yucca shidigera* (Yucca; gray bars), or inoculated with media from cultures chronically treated with Yucca and receiving no treatment (checkered bars) or a 10X concentration of Yucca (black bars). [a, b, c, d] Least squares means with different superscript within day differ (P < 0.05).

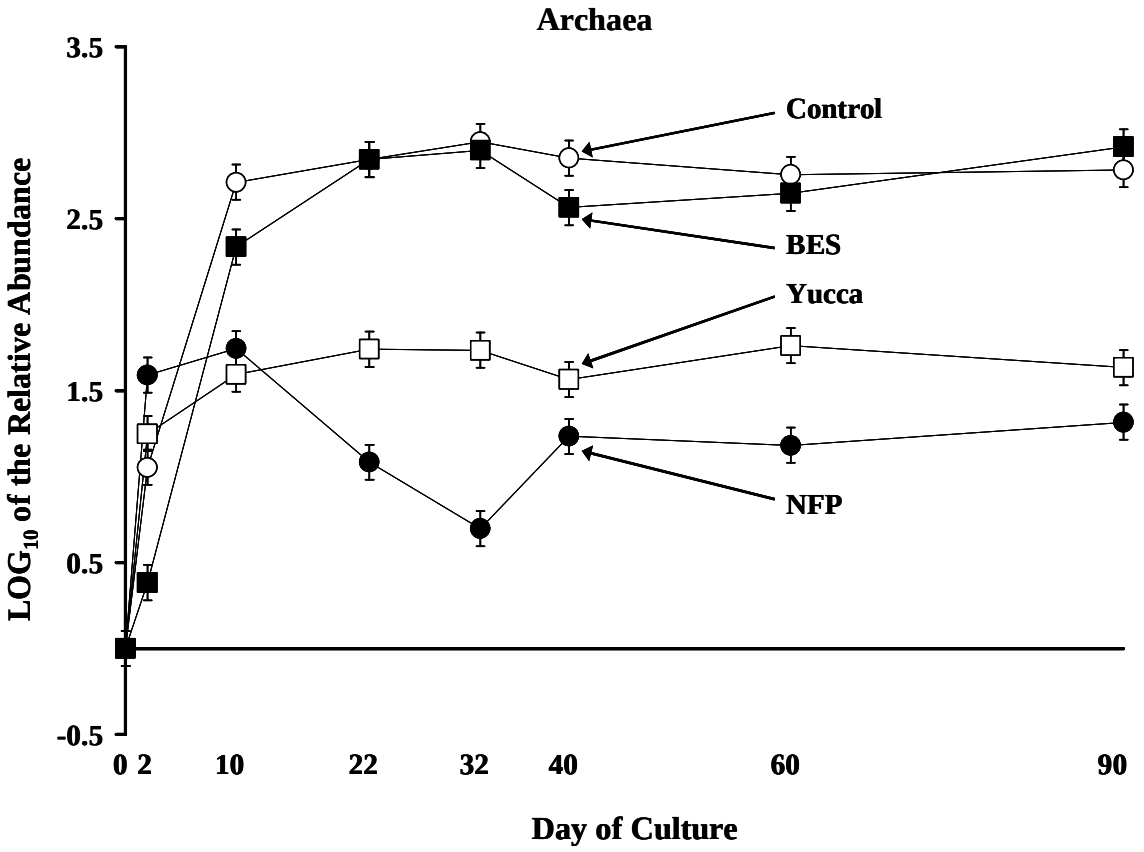


Figure 2.5. Least squares means ± SEM for the relative abundance of Archaea in control cultures (—○—) or those chronically treated with a nitrofuranyl derivative of para-aminobenzoate (NFP; —●—), an extract from the plant *Yucca shidigera* (Yucca; —□—), or 2-bromoethansulfonate (BES; —■—). Within day of culture, significant ($P < 0.05$) contrasts relative to Control: NFP (days 2, 10, 22, 32, 40, 60, and 90), Yucca (days 10, 22, 32, 40, 60, and 90), and BES (days 2 and 10).

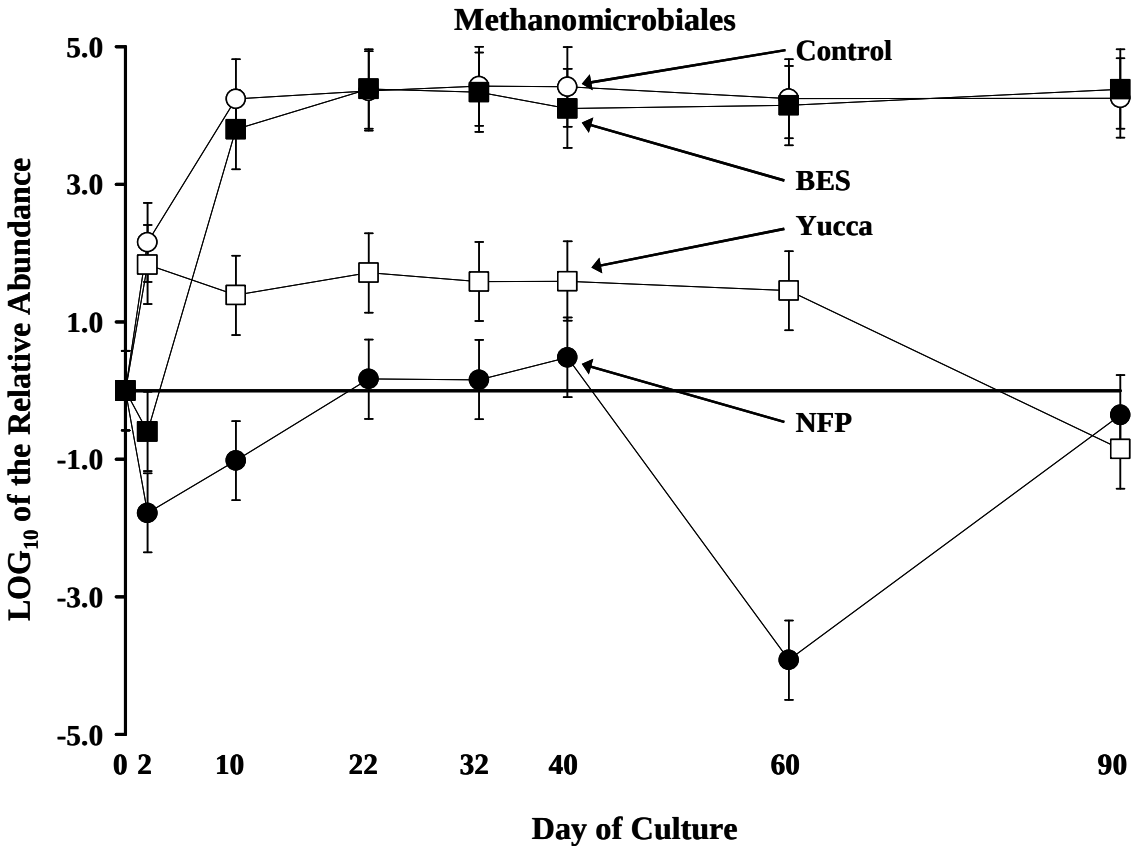


Figure 2.6. Least squares means ± SEM for the relative abundance of Methanomicrobiales in control cultures (—○—) or those chronically treated with a nitrofuranyl derivative of para-aminobenzoate (NFP; —●—), an extract from the plant *Yucca shidigera* (Yucca; —□—), or 2-bromoethansulfonate (BES; —■—). Within day of culture, significant ($P < 0.05$) contrasts relative to Control: NFP (days 2, 10, 22, 32, 40, 60, and 90), Yucca (days 10, 22, 32, 40, 60, and 90), and BES (day 2).

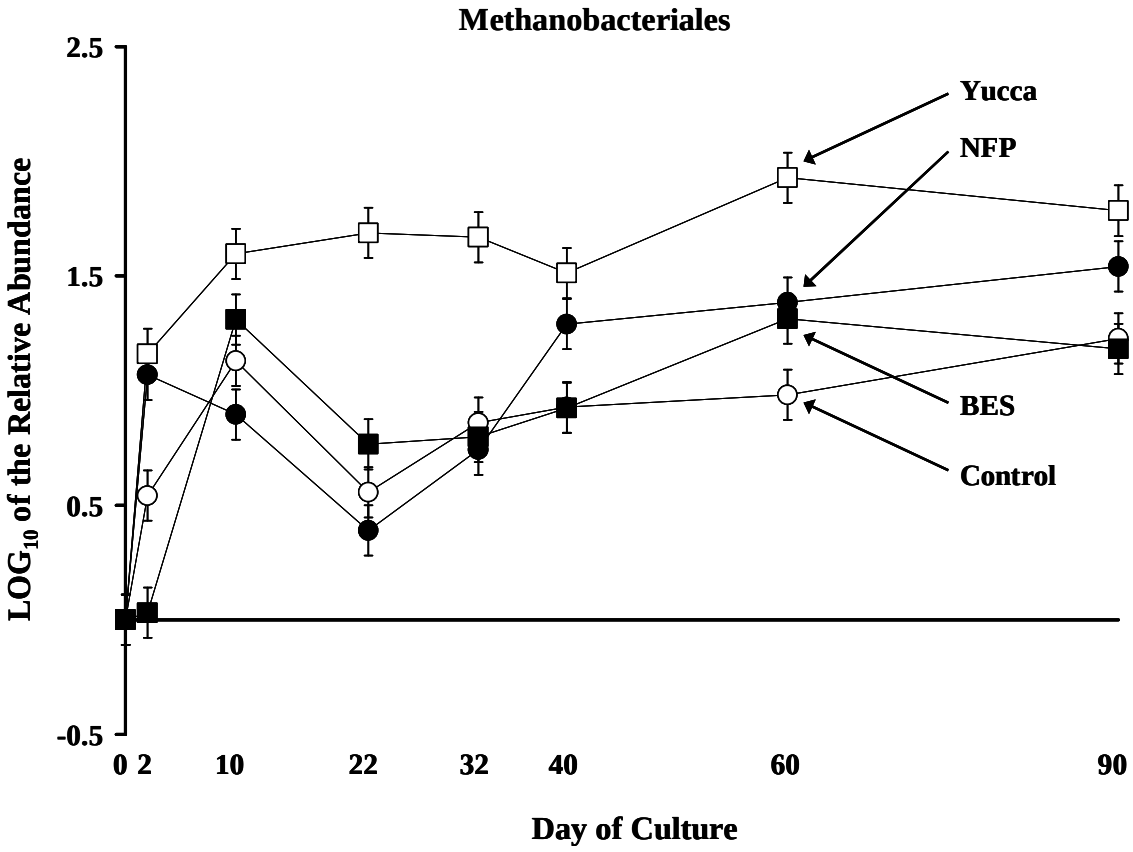


Figure 2.7. Least squares means ± SEM for the relative abundance of Methanobacteriales in control cultures (—○—) or those chronically treated with a nitrofuranyl derivative of para-aminobenzoate (NFP; —●—), an extract from the plant *Yucca shidigera* (Yucca; —□—), or 2-bromoethansulfonate (BES; —■—). Within day of culture, significant ($P < 0.05$) contrasts relative to Control: NFP (days 2, 10, 40, 60, and 90), Yucca (days 2, 10, 22, 32, 40, 60, and 90), and BES (day 2).

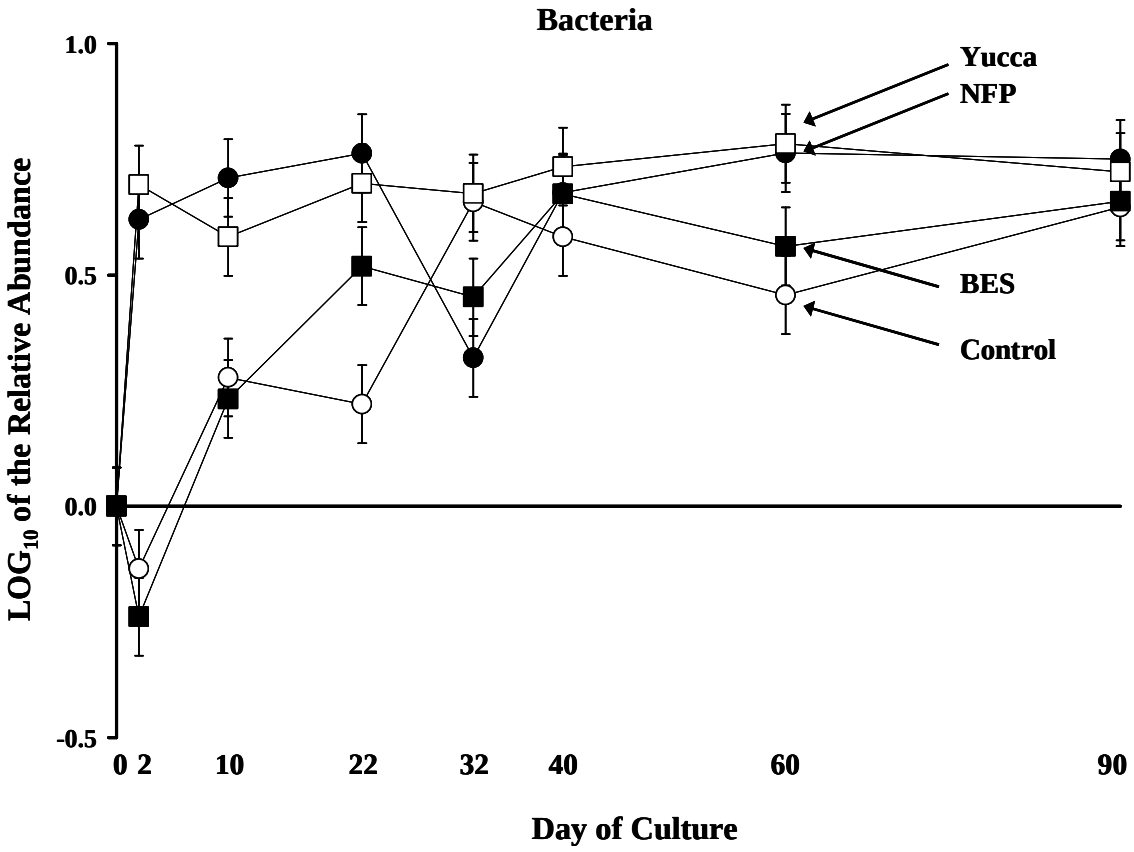


Figure 2.8. Least squares means ± SEM for the relative abundance of Bacteria in control cultures (—○—) or those chronically treated with a nitrofuranyl derivative of para-aminobenzoate (NFP; —●—), an extract from the plant *Yucca shidigera* (Yucca; —□—), or 2-bromoethansulfonate (BES; —■—). Within day of culture, significant ($P < 0.05$) contrasts relative to Control: NFP (days 2, 10, 22, 32, 60, and 90), Yucca (days 2, 10, 22, and 60), and BES (day 22).

CHAPTER III

Ruminal methane production is differentially affected following the replacement of dietary forage or concentrate with distillers dried grains plus solubles

ABSTRACT: Our objectives were to determine: 1) the influence on in vitro methane (CH_4) production of replacing either brome hay or corn with distillers dried grains plus solubles (DDGS) and 2) the influence on ruminal methanogenesis of replacing either forage or corn with DDGS in low- and high-concentrate diets, respectively. In vitro experiments 1 and 2 were conducted by providing ruminal cultures with 0, 25, 50, 75, or 100% DDGS with the balance being either brome hay or corn, and incubated at 39ºC for 22 h. Sheep experiment 1 used a replicated Latin square design. Intact (n = 9) and ruminally fistulated (n = 3) lambs were offered a brome hay-based ration (1% BW) that contained 30% corn bran (BRAN), 30% DDGS (30DDGS), or 30% DDGS and 30% corn bran (DDGS+BRAN). Sheep experiment 2 used a cross-over design. Intact lambs (n = 9) were offered a ration (3% BW) containing 71% corn and 2.2% corn oil (CORN), or one in which DDGS replaced corn for 30% of the diet (CORN/DDGS). Following adaptation to diet, exhaled gas was collected (2 d) to determine CH_4 production by the SF_6 tracer technique. Feces and orts were collected (5 d) to determine digestibility, and ruminal fluid was collected (1 d) for determination of pH and VFA concentrations. Real-time PCR was used to monitor the changes in ruminal microbe populations. In vitro replacement of brome hay with DDGS decreased ($P < 0.01$) the amount of CH_4 produced per milligram of DM. Replacement of corn with DDGS in vitro increased ($P < 0.01$) the amount of CH_4 produced per milligram of DM. For sheep experiment 1, we detected a

main effect of diet ($P < 0.01$) on CH_4 production rate per kilogram of digested DM. The BRAN animals produced 158 mmol $\bullet h^{-1} \bullet kg^{-1}$, which was greater ($P < 0.05$) than that of the 30DDGS or DDGS+BRAN animals (135 and 113 mmol $\bullet h^{-1} \bullet kg^{-1}$, respectively). However, the relative abundance of Archaea or Methanobacteriales was not affected by diet, but that of Methanomicrobiales was greater ($P < 0.05$) for the DDGS+BRAN diet compared to the BRAN or 30DDGS diets. For sheep experiment 2, replacement of corn with DDGS increased ($P < 0.05$) CH_4 production rate per kilogram of digested DM from 68.3 to 88.9 mmol $\bullet h^{-1} \bullet kg^{-1}$. We conclude that the replacement of forage with DDGS is an effective strategy for decreasing ruminal CH_4 production but simultaneously replacing corn and corn oil with the ethanol production coproduct enhances ruminal methanogenesis.

INTRODUCTION

Ruminal methanogenesis accounts for a 2 to 12% loss of feed GE (Johnson and Johnson, 1995), and retention of this energy would be an addition to the amount of energy available for production. Methane (**CH_4**) is a potent greenhouse gas and anthropogenic sources are responsible for 55to70% of total CH_4 emissions (IPCC, 2001). Emissions from enteric fermentation represent about 20% of total CH_4 emissions from anthropogenic activities, with cattle being the largest emitters (EPA, 2007). Feeding strategies that attenuate ruminal methanogenesis may increase the efficiency of ruminal fermentation and aid in decreasing atmospheric CH_4 concentrations.

Coproducts from the production of corn-ethanol are becoming important feed products as their supply has recently increased (Rausch and Belyea, 2006a). When

incorporated into a finishing diet, wet distillers grains (Larson et al., 1993; Ham et al., 1994) and distillers dried grains plus solubles (**DDGS**) (Ham et al., 1994) have a NE value greater than corn. The increased net energy values may partly be attributable to a decrease in CH_4 production.

Production of CH_4 per unit of digestible DM decreases as the proportion of grain in the diet increases (Johnson and Johnson, 1995; Benchaar et al., 2001; Boadi et al., 2004). Methanogenic potential in the rumen is greater for cell wall carbohydrates than for starch (Johnson et al., 1996). In addition, dietary fat also suppresses CH_4 production (Dong et al., 1997; Machmuller and Kreuzer, 1999; Dohme et al., 2000). Compared to DDGS that has an NDF and ether extract (**EE**) content of 46 and 10%, respectively, brome hay possesses a lesser ether extract (2%) content and greater NDF (58%) content, but corn possesses a lesser NDF (11%) and EE (4%) content (NRC, 2000). Our objective was to determine the influence on ruminal methanogenesis of replacing either corn or forage with DDGS in high- and low- concentrate rations, respectively.

MATERIALS AND METHODS

In vitro Experiments

Ruminal sampling and animal care. Ruminal fluid was collected from one ruminally fistulated heifer fed a mixed forage and concentrate diet (Table 3.1). Fluid was collected using a suction strainer, strained through four layers of cheesecloth, and immediately transported to the laboratory. The procedures used for ruminal sampling were approved by the University of Nebraska's Institutional Animal Care and Use Committee (protocol #04-05-032).

Cultures. All feedstuffs used as a substrate for in vitro fermentation were ground in a Wiley mill (Thomas Scientific, Philadelphia, Pennsylvania) to pass a 1-mm screen. Multiple substrate combinations were provided to cultures (n = 4 per combination) at a rate of 10 mg/mL and composed of 100, 75, 50, 25, or 0% DDGS with the balance being either brome hay or fine ground corn (in vitro experiments 1 and 2, respectively). Cultures were composed of 6 mL of ruminal fluid, 12 mL of a modified McDougall's buffer (McDougall, 1948) (0.2 M $NaHCO_3$, 0.02 mM Na_2HPO_4, 8 mM KCl, 8 mM NaCl, 0.5 mM $MgSO_4$, and 1 mM $CaCl_2$), 12 mL of distilled H_2O, 60 mg trypticase, 30 µg of resazurin, 38 µL of a micro mineral solution (0.5 M $MnCl_2$, 0.04 M $CoCl_2$, and 0.3 M $FeCl_3$), and 30 mg of Na_2S. The concentration of $NaHCO_3$ was increased to 0.5 M for experiment 2 to buffer a drop in pH that would likely inhibit methanogenesis. The culture medium was gassed with CO_2 to create oxygen-free medium and then added to 37 mL glass vials, which contained the respective substrate combination, as CO_2 was projected into each vial. The vials were then sealed, purged with CO_2, pressurized to 100 kPa above atmospheric pressure, and placed in a shaking incubator (39°C) for 22 h. Following incubation, cultures were cooled to room temperature and the headspace pressure was measured using a micromanometer. Concentration of CH_4 was assayed with a Varian 3700 gas chromatograph (Varian, Inc., Palo Alto, California) fit with a thermal conductivity detector and a silica gel 60/80 grade packed column. Injector, detector, and column temperatures were 120, 120, and 60°C, respectively. Final pH of the culture fluid was recorded for only the DDGS/fine ground corn experiment. Medium

from each culture was then centrifuged. Assay of VFA concentration is described below. IVDMD was determined by filtration and subsequent drying of the filter (60^{o}C) for 48 h.

Lamb Experiments

Animal care. The procedures used for both in vivo experiments were approved by the University of Nebraska's Institutional Animal Care and Use Committee (protocol #05-07-046D).

Lamb experiment 1. The objectives were to determine: 1) the effect of replacing brome hay with DDGS on ruminal CH_4 production and 2) if any observed effect may be attributed to the non-fiber (protein + fat) versus the corn bran component of the DDGS. The 3 brome hay-based diets formulated to achieve these objectives were a diet containing corn bran (60% brome hay and 30% corn bran; **BRAN**), a diet in which the corn bran was replaced with DDGS (60% brome hay and 30% DDGS; **30DDGS**), and a diet in which brome hay was replaced with DDGS (30% brome hay, 30% corn bran, and 30% DDGS; **DDGS+BRAN**). The comparison of the BRAN diet versus the DDGS+BRAN diet would test the effects of replacing brome hay with DDGS whereas the comparisons of the BRAN diet versus the 30DDGS diet or the 30DDGS diet versus the DDGS+BRAN diet would isolate the effects of either the non-fiber or the corn bran components, respectively, of the DDGS. Nine intact crossbred lambs (23.5 ± 2.8 kg) and 3 ruminally cannulated crossbred lambs (33.7 ± 7.8 kg) were blocked based on the presence or absence of a cannula and assigned randomly to receive a sequence of diets in a replicated 3 × 3 Latin square design. Diets were offered once daily at 0800. Prior to sheep experiment 1, feed refusals were collected to determine ad libitum intake. Average

ad libitum DM intake for the three diets was 1.17% BW. To minimize ingredient sorting, lambs were limit-fed for the duration of the experiment at 1.0% BW. Periods were 14 d with 9 d of adaptation followed by 5 d of collecting orts and feces for determination of DM digestibility. Exhaled gas was collected from 0800 to 1400 and CH_4 production rate was determined on d 13 and 14 of each period for the intact lambs and d 12 and 13 of each period for the cannulated lambs. On d 14 of each period ruminal fluid was collected from the cannulated lambs at 0800, 1200, 1600, and 2000. The pH of the samples was recorded and the samples were immediately frozen for later VFA analysis.

Lamb experiment 2. The objective was to determine the effect of simultaneously replacing corn and corn oil with DDGS on ruminal CH_4 production. The 2 corn-based diets formulated to achieve this objective were a diet containing 71.4% corn, 2.2% corn oil, and 26.4% forage and supplement (**CORN**) and one in which DDGS replaced corn for 29.9% of the diet (43.7% corn, 29.9% DDGS, and 26.4% forage and supplement; **CORN/DDGS**). Following sheep experiment 1, the 9 intact lambs were assigned randomly to receive a sequence of diets in a crossover design. Diets were pelleted and offered (3% BW) twice daily at 0800 and 1600. Lambs were adapted to grain by feeding 50, 40, 35, and 30% forage and supplement (DM basis) replaced by concentrate (corn or corn + DDGS) for 4, 4, 4, and 2 d, respectively, prior to the commencement of feces collection. Periods were 19 d with 14 d of adaptation and 5 d of collecting orts and feces for determination of DM digestibility. Exhaled gas was collected from 0800 to 1400 and CH_4 production rate was determined on d 17 and 18 of each period. Ruminal fluid was

collected via the esophagus at 1200 on d 19. Immediately upon collection the pH of the sample was recorded, and the samples were frozen and stored for later VFA analysis.

Gas collection and analysis. Production rate of CH_4 was determined by procedures modified from those developed by Johnson et al. (1994). Brass permeation tubes containing 609 to 735 mg of SF_6 and with known release rates (1,875 to 2,639 ng of SF_6/min) were placed in the rumen of intact (via the esophagus) and cannulated (via the fistula) lambs 1 d prior to the first gas collection period. On each collection day exhaled gas was drawn into evacuated (50 mmHg) PVC collection canisters for a 6-h period. Gas was drawn through 46 cm of capillary tubing (128 µm i.d.; Alltech Associates, Inc., Deerfield, Illinois) with an in-line 15-µm filter (Swagelok, Solon, Ohio) and nosepiece that rested above the animal's mouth and nose (Appendix II). Analysis of CH_4 was conducted utilizing a HP 5890 gas chromatograph (Agilent Technologies, Inc., Palo Alto, California) fit with a flame ionization detector and Propak Q 80/100 column (Restek, Bellefonte, Pennsylvania). Column, injector, and detector temperatures were 60, 100, and 150ºC, respectively and N_2 (20 mL/min) was used as the carrier gas. Analysis of SF_6 was conducted utilizing a HP 6890 gas chromatograph (Agilent Technologies, Inc.) fit with a micro-electron capture detector and HP-PLOT Molesieve 5A column (Agilent Technologies, Inc.). Column, injector, and detector temperatures were 40, 35, and 250ºC, respectively, and N_2 (1 mL/min) was used as the carrier gas.

Feedstuff and VFA Analyses

Feedstuff analyses. The DM concentration of diet samples, orts, and feces was determined by drying samples in a 60ºC oven for a minimum of 48 h. The NDF content

of diet samples was determined according to the procedure of Van Soest et al. (1991) with the filter bag technique using the ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, New York) following a pre-extraction with acetone, and the inclusion of sodium sulfite and heat stable alpha-amylase. The CP content (%N × 6.25) of diet samples was determined using a combustion-method N analyzer (Leco FP-528, Leco Corp., St. Joseph, MI) according to method No. 990.03 (AOAC, 1995). Starch and EE concentrations were analyzed using methods No. 996.11 and No. 920.39 (AOAC, 1995), respectively.

VFA analysis. The concentration of VFA was determined as previously described (Erwin et al., 1961) utilizing a HP 5890 gas chromatograph (Agilent Technologies, Inc.) fit with a Supelco 12144 column.

Relative Quantification of Microbes

DNA extraction. Ruminal fluid collected from cannulated lambs during the first period of sheep experiment 1 was immediately frozen (-80ºC) and DNA was extracted at a later date. For the remainder of the samples from sheep experiments 1 and 2, total genomic DNA was extracted from fresh ruminal fluid that had been strained through 4 layers of cheesecloth utilizing the RBB+C method (Yu and Morrison, 2004), which employs a QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, California) following repeated bead beating steps to disrupt cell membranes. The quality and quantity of DNA samples were determined with gel electrophoresis and by spectrophotometry, respectively.

Real-time PCR. The relative presence of targets for 2 domain-specific (Bacteria and Archaea) and 4 order-specific (Methanobacteriales, Methanomicrobiales, Methanococcales, and Methanosarcinales) primer and probe sets (Yu et al., 2005) was determined for each sample in duplicate with real-time PCR utilizing an AB7700 (Applied Biosystems, Foster City, California). Each reaction well contained 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 4.2 µL of community DNA, 1.25 µL of the forward and reverse primers (final concentration, 500 nM), 0.4 µL of the corresponding probe (final concentration, 150 nM), and nuclease-free water to a final volume of 25 µL. The following reaction conditions were applied to each well: an initial 2-min incubation at 50ºC; a 10-min incubation at 95ºC; and 45 cycles of denaturation at 95ºC for 10 s, annealing at 55ºC for 30 s, and extending at 60ºC for 1 min.

An optimal in-well DNA concentration was established for each primer and probe set to maximize the DNA present in each well and allow for the detection of targets present in small amounts while avoiding the inhibitory effects associated with most DNA samples (Gallup and Ackermann, 2006). The threshold cycle (**Ct**) values for each primer and probe set were determined with a test plate containing serially diluted samples from a pooled stock. This stock was created by combining aliquots of each experimental sample. The Ct values were then plotted against the $LOG_{(10)}$ of the dilution factor, and the linear portion of this plot indicated the concentrations of DNA at which inhibition was absent. The DNA concentration used for each primer and probe set was 60% less than the maximum determined to avoid inhibition of the PCR reaction. The optimal in-well DNA concentrations for the primer and probe sets targeting Archaea,

Methanobacteriales, Methanomicrobiales, and Bacteria were 1.396, 0.558, 0.265, and 0.089 ng/µL, respectively. Four samples containing decreasing concentrations of DNA and diluted from the stock solution were tested in duplicate on each PCR plate. The difference between the average Ct value of these standards on all plates and the average Ct value for the 4 standards on each plate was added to the Ct of all samples on each respective plate to neutralize inter-plate variation. The Ct of each plate's four standards was plotted on the $LOG_{(10)}$ of the dilution factor. The slope of this line indicated the efficiency of amplification (**E_{AMP}**). The Ct value for each unknown was then standardized to a common denominator with equation 2.1.

Calculations

In vitro experiments. The amount of CH_4 present (µmol) following incubation was divided by [(milligrams of DM added to each vial) × (mean treatment IVDMD)] to obtain CH_4 produced per milligram of digested DM. Volatile fatty acid concentration (mM) of initial fermentation media was subtracted from VFA concentration of all cultures following fermentation (mM) to determine VFA production and this was divided by [(grams of DM added to each vial) × (treatment IVDMD)] to determine VFA production per unit of digested DM (mmol/g). Total kilocalories produced in the form of VFA was calculated by summing the production of acetate, propionate, and butyrate multiplied by 209.4, 367.2, and 524.3, respectively, as these are the energy values (kcal) of the individual VFA (Owens and Goetsch, 1988).

Lamb experiments. Emission rates of CH_4 were calculated by multiplying the permeation rate of SF_6 by the ratio of the concentrations of CH_4 and SF_6 present in the

collections canisters (Johnson et al., 1994). Emission rates of CH_4 per kilogram of digested DM were calculated by dividing the CH_4 emission value by the product of the amount of DM consumed on the day of collection multiplied by the mean digestibility for the diet.

Real-time PCR. For each primer and probe set, the average Ct value acquired either from samples obtained at 0800 during the period the animals were offered the BRAN diet (sheep experiment 1) or an arbitrary animal offered the CORN diet (sheep experiment 2) was used as a "Reference Ct" and a value of 1.0 relative abundance units was assigned to these samples. The abundance of target for each primer and probe set for all other samples was calculated with equation 2.2.

Statistical Analysis

All data were analyzed utilizing the MIXED procedure of SAS (SAS Inst., Inc., Cary, North Carolina). In vitro experiment 1 and 2 data were analyzed by direct regression. An initial response curve was fit including linear and quadratic effects of DDGS inclusion level. Only the significant effects were retained in the reported response curve model.

Lamb experiment 1 data were analyzed as a replicated 3 × 3 Latin square design. Animal within period was the experimental unit. The model included the fixed effects of square, period, dietary treatment, and day within period and the random effect of animal. All of the real-time PCR data were $LOG_{(10)}$ transformed prior to analysis to account for non-constant variances as indicated by the Shapiro-Wilk test for normality. Because VFA concentrations and the relative abundance of microbes were determined for samples

collected from the same animals on an individual day within each period a repeated measures covariance structure was used. The AR(1) covariance structure was selected based on the Akaike information criterion. Least squares means were separated using the protected least significant difference test when a significant overall treatment *F*-test ($P < 0.05$) was detected. CH_4 production data from 1 intact animal on 1 d in the second period and 2 d in the third period were excluded from the analysis because the vacuum in that animal's collection canister failed to dissipate during the collection period. Digestibility of DM and CH_4 production data from a separate intact animal in the third period were excluded from the analysis as the animal was observed consuming an excessive amount of wool. Digestibility data from 2 cannulated animals during the second period were excluded from the analysis because of a loss of an unknown amount of ruminal contents during the fecal collection period.

Lamb experiment 2 data were analyzed as a 2-period cross-over design. Animal within period was the experimental unit. The model included the fixed effects of dietary treatment, period, and day within period. Animal was a random effect. The real-time PCR data from lamb experiment 2 were also $LOG_{(10)}$ transformed prior to analysis to account for non-constant variances as indicated by the Shapiro-Wilk test for normality. Because VFA concentrations and the relative abundance of microbes were determined for samples collected from the same animals on an individual day within each period, a repeated measures covariance structure was used. The AR(1) covariance structure was selected based on the Akaike information criterion. Least squares means were separated using the protected least significant difference test when a significant overall treatment *F*-

test ($P < 0.05$) was detected. The CH_4 production data from 1 animal on 1 day were excluded from the analysis because the vacuum in that animal's collection canister failed to dissipate during the collection period.

RESULTS

In vitro Experiment 1

Nutrient content of the brome hay and DDGS is outlined in Table 3.2. Total CH_4 produced by cultures decreased ($P < 0.01$) but IVDMD increased ($P < 0.01$) as DDGS replaced brome hay (Figure 3.1). Per milligram of digested DM CH_4 production decreased ($P < 0.01$) with increasing inclusion rate of DDGS. Replacement of brome hay with DDGS decreased ($P < 0.01$) acetate production but increased ($P < 0.01$) propionate and butyrate production.

In vitro Experiment 2

Nutrient content of the fine ground corn is outlined in Table 3.2. Replacement of fine ground corn with DDGS decreased ($P < 0.01$) IVDMD but increased ($P < 0.01$) CH_4 production per milligram of digested DM (Figure 3.2). As DDGS replaced fine ground corn, acetate production increased ($P < 0.01$) but propionate and butyrate production decreased ($P < 0.01$). Furthermore, the kilocalories produced in the form of VFA decreased ($P = 0.02$) as DDGS replaced fine ground corn.

Lamb Experiment 1

Composition and nutrient content of the diets is outlined in Table 3.3. Diet did not affect DMI but DM digestibility was greater ($P < 0.01$) for the DDGS+BRAN diet compared to both the BRAN and 30DDGS diets (Table 3.4). We detected a main effect

of diet ($P < 0.01$) on CH_4 production rate per kilogram of digested DM. The BRAN diet resulted in a greater rate of CH_4 production per kilogram of digested DM compared to either the 30DDGS diet ($P = 0.02$) or the DDGS+BRAN diet ($P < 0.01$), and the 30DDGS diet resulted in a greater ($P = 0.03$) CH_4 production rate than did the DDGS+BRAN diet. Diet and time did not interact to affect any variables associated with ruminal fluid, but the main effect of diet ($P < 0.01$) was detected for propionate concentrations. Animals receiving the BRAN diet had greater ruminal propionate concentrations compared to 30DDGS ($P < 0.01$) and DDGS+BRAN ($P = 0.04$) animals. Animals receiving the 30DDGS diet had lesser ($P = 0.05$) propionate concentrations compared to DDGS+BRAN animals. There tended to be a main effect of diet ($P = 0.08$) on acetate concentrations. The BRAN diet resulted in greater ($P = 0.03$) acetate concentrations compared the DDGS+BRAN diet, but no difference existed when comparing the 30DDGS diet to either the BRAN or DDGS+BRAN diets. Differences between diets were not observed in pH or butyrate concentrations. There was a tendency ($P = 0.06$) for diet to affect the relative abundance of total Archaea (Figure 3.3). Relative to the BRAN and 30DDGS diets, the DDGS+BRAN diet resulted in a greater ($P < 0.05$) relative abundance of Methanomicrobiales.

Lamb Experiment 2

Composition and nutrient content of the diets is outlined in Table 3.5. Intake of dry matter was greater ($P < 0.01$) for the CORN diet but no difference in digestibility was detected (Table 3.6). Replacement of corn with DDGS increased ($P = 0.01$) the CH_4 production rate per kilogram of digested DM. The pH of collected ruminal fluid tended

to be lesser ($P = 0.06$) for CORN/DDGS animals compared to CORN animals, but no differences were detected in the concentrations of acetate, propionate, or butyrate. The CORN/DDGS diet tended ($P = 0.06$) to increase the relative abundance of ruminal bacteria (Figure 3.4).

DISCUSSION

In vitro Experiments

Replacement of brome hay with DDGS as a substrate for ruminal cultures decreased CH_4 production but replacement of corn with DDGS increased total CH_4 production. These effects may be a function of the carbohydrate being fermented (fiber vs. starch), fat, or a combination of the two factors. In vivo fermentation of fiber results in a greater amount of CH_4 compared to the fermentation of starch (Johnson et al., 1996). This has been confirmed in vitro by Eun et al. (2004) assuming an adjustment for quantity of substrate fermented. It is unlikely that the nature of the carbohydrate being fermented in the current in vitro experiment is fully responsible for the observed response. The large amount of EE present in the DDGS relative to either the brome hay or the corn may also have influenced CH_4 production. Addition of fat to ruminal cultures may inhibit methanogenesis (Dohme et al., 2000; Soliva et al., 2003). The combined effects of carbohydrate and EE are supported by the quadratic response observed for CH_4 produced per milligram of digested DM when DDGS replaced either brome hay or corn. It is possible that as up to 50% of the brome hay is replaced with DDGS the change in CH_4 may be attributed to a lesser amount of NDF, and when >50% of the substrate is composed of DDGS the greater EE inhibits methanogenesis. Replacement of up to 50%

of the corn with DDGS increases CH_4 production, perhaps because starch is replaced with NDF. As >50% of the corn is replaced the additional EE likely offsets the effect of replacing starch with structural carbohydrates.

The proportion of individual VFA produced by ruminal cultures has been reported to be a function of culture pH (Peters et al., 1989; Russell, 1998). Because of the strongly buffered culture media utilized it is unlikely that either DDGS or brome hay would have resulted in a differential pH and this is supported by pH data from the in vitro experiment in which fine ground corn was replaced with DDGS. In concurrence with our data, Russell (1998) reported that fermentation of a fibrous substrate increases the acetate to propionate ratio and CH_4 production compared to fermentation of a starchy substrate.

Lamb Experiment 1

Replacement of either brome hay (BRAN diet vs. DDGS+BRAN diet) or corn bran (BRAN diet vs. 30DDGS diet) with DDGS in a sheep's diet decreased CH_4 production per kilogram of digested DM. Such an effect was expected as substitution of DDGS for both ingredients resulted in lesser NDF and greater EE contents. One millimole of CH_4 has a heat of combustion of 0.2108 kcal (Owens and Goetsch, 1988). Therefore, during the 6-h collection period the BRAN, DDGS, and DDGS+BRAN diets would have resulted in the loss of 37, 32, and 31 kcal, respectively, in the form of CH_4. Compared to the BRAN diet the DDGS+BRAN diet contained DDGS in place of brome hay. Because DDGS contains a greater amount of DE compared to brome hay (3.84 vs. 2.43 Mcal/kg) (NRC, 1985) the effect of each feedstuff on ruminal CH_4 production is exacerbated when examined as a percentage of DE lost as CH_4. The increased EE

content of the diet containing DDGS would have likely suppressed CH_4 production. Sheep offered a forage-based diet produced 22% less CH_4 as myristic acid was used to increase dietary EE from 1.9 to 5.9% (Machmuller et al., 2003b). However, in vitro data indicate that not all fatty acids inhibit methanogenesis to the extent of myristic acid (Dohme et al., 2001). The EE content of the DDGS+BRAN and 30DDGS diets were 2.8 and 2.7 percentage points, respectively, greater than that of the BRAN diet. With regard to fatty acids, linoleic (C18:2) is one of the most potent inhibitors of methanogenesis (Dohme et al., 2001), but linoleic acid comprises only 54% of the EE component of corn (USDA ARS, 2005). Because the EE encompasses a number of fatty acids, and not just those that inhibit methanogenesis, it is possible that this dietary component is not fully responsible for the decrease in CH_4 production following replacement of brome hay or corn with DDGS. Benchaar et al. (2001) estimated that when comparing alfalfa of midbloom and vegetative maturities (47 and 31% NDF, respectively) the latter would result in 15% less CH_4 when expressed as a percent of DE. Therefore, a decrease of 11 and 14 percentage points of NDF content likely contributed to the observed 29 and 15% , respectively, decrease in CH_4 produced per kilogram of digested DM when comparing the substitution of DDGS for brome hay or corn bran.

The second objective for lamb experiment 1 was to determine if an observed effect on ruminal CH_4 production could be attributed to the non-fiber vs. the corn bran component of the DDGS. The non-fiber component appeared to inhibit ruminal CH_4 production as indicated by the difference between the BRAN and 30DDGS diets, but the effect of the addition of solubles was confounded with the effect of a decreased NDF

content. Consequently, we cannot exclude the possibility that fiber, in addition to non-fiber components, contributes to the effect of DDGS on methanogenesis.

Replacement of corn bran with DDGS (BRAN diet vs. 30DDGS diet) decreased propionate concentrations, and replacement of brome hay with DDGS (BRAN diet vs. DDGS+BRAN diet) also decreased propionate concentrations. Despite the lack of a significant diet effect on acetate or butyrate concentrations the trend for both variables is similar across treatments to that of propionate concentrations. Some caution is warranted when comparing VFA concentrations with CH_4 production. Concentration of VFA is a function of not only production rate, but also absorption and passage rates, and ruminal volume. Given that 30DDGS enhanced digestibility it is unlikely that propionate concentration reflects a diminished production rate. More likely, ruminal VFA were diluted due to enhanced liquid passage. We surmise that replacement of brome hay with DDGS increased the osmotic load within the rumen, which would have increased passage rate.

Lamb Experiment 2

Similar to what was observed in vitro, replacement of corn with DDGS increased CH_4 production in vivo. When the amount of energy lost in the form of CH_4 is calculated, the CORN and CORN/DDGS diets resulted in losses of 42 and 50 kcal, respectively, during the 6-h collection period. Because both corn and DDGS have identical DE contents (3.84 Mcal/kg) the difference in the fractional DE losses of CH_4 between the treatments would be similiar to the difference in CH_4 emissions. The effect of simultaneously replacing corn and corn oil with DDGS on ruminal methanogenesis has

not been previously reported. However, data from many studies indicate that CH_4 production is affected by the relative proportions of soluble and structural carbohydrates within a diet (Johnson et al., 2000). Guan et al. (2006) adjusted the ratio of structural to nonstructural carbohydrates to create low- and high-concentrate diets. The high-concentrate diet resulted in a lesser percentage of feed GE lost as CH_4. Further to this point, Benchaar et al. (2001) concluded that a diet composed of a starchy concentrate (20% starch and 23% NDF) would result in 22% less DE lost as CH_4 compared to a diet containing a fibrous concentrate (2% starch and 31% NDF). Therefore, the 51% greater NDF content of the CORN/DDGS diet compared to the CORN diet is likely responsible for the 30% increase in CH_4 produced per kilogram of digested DM upon DDGS inclusion. Interpretation of the CH_4 production data should consider that the digestibility value used in the denominator reflects total tract digestibility rather than ruminal digestibility. Because DDGS contains a greater proportion of UIP than corn, ruminal digestibility likely was lesser for the CORN/DDGS diet compared to the CORN diet. Therefore, the greater CH_4 production rate per kilogram of DM digested in the rumen of the CORN/DDGS diet compared to the CORN diet may be understated.

Fatty acids present in corn oil suppress methanogenesis (Dohme et al., 2001). Oil was added to the CORN diet and not the DDGS diet. We don't know how methanogenesis is influenced by the endogenous oil in DDGS relative to the added oil in the corn diet. The endogenous oil in DDGS may be less exposed to biohydrogenation and therefore may serve as less of a sink for reducing equivalents used for CH_4 production. However, if all of the unsaturated fatty acids in the corn oil added to the

CORN diet became saturated before leaving the rumen and none of the fatty acids in the DDGS of the CORN/DDGS diet became saturated, this alternative for disposing of reducing equivalents would only account for 3.9 mmol•h^{-1}•kg^{-1} CH_4 production. Therefore, under the most extreme circumstances, decreased biohydrogenation of the fatty acids associated with DDGS could only account for a 5% difference in CH_4 production.

We expected propionate concentrations to decrease following the replacement of corn with DDGS, but no effect was observed. The absence of an effect on VFA concentrations when DDGS replaced corn for 30% of the diet is similar to a previous report in which DDGS was used to replace corn for 40% of the diet (Ham et al., 1994).

Ruminal Microbiology

Assumptions made for interpretation of the real-time PCR include: 1) the efficiency of DNA extraction was similar for all samples and 2) ruminal volume was not affected by treatment. These are considered to be safe assumptions.

Investigators have reported that methanogens of the order Methanobacteriales are the most abundant in the ruminal environment (Sharp et al., 1998; Tokura et al., 1999; Skillman et al., 2006; Nicholson et al., 2007; Wright et al., 2007). Also, Methanomicrobiales has been reported by others to be the most (Yanagita et al., 2000; Tajima et al., 2001) or second most (Sharp et al., 1998; Wright et al., 2007) abundant in the ruminal environment and this may depend on host species (Lin et al., 1997). Methanobacteriales was the most abundant order in our lambs receiving a concentrate-based diet, as Methanomicrobiales was not detected. In the lambs receiving a forage-

based diet, Methanobacteriales was more static than Methnomicrobiales as brome hay and corn bran was replaced with DDGS. We were unable to detect the presence Methanosarcinales and Methanococcales in any of our samples.

The population of methanogens present in animals receiving different diets has been surveyed (Lin et al., 1997; Wright et al., 2007), but a formal test of the effect of diet on different classifications of methanogens has not been reported. Despite a decrease in CH_4 production following the replacement of corn bran or brome hay with DDGS, we did not observe a difference in the relative abundance of total ruminal Archaea. Even though the authors did not analyze data in such a way to detect differences among means, Lin et al. (1997) did report that as a percentage of the total amount present, the amount of small subunit ribosomal RNA of Archaea was 2.4 ± 0.2, 1.6 ± 0.5, and 2.1 ± 0.7 in samples obtained from bovine animals offered a diet of 0, 50, and 70% concentrate, respectively. Increasing the percentage of concentrate in a diet will decrease CH_4 production per unit of digested DM, yet an obvious decrease is not present in the abundance of Archaea observed by Lin et al. (1997). Therefore, our results are consistent with previous observations in that that the relative abundance of total Archaea is not a sound indicator of CH_4 production.

Both in vitro and in vivo, replacement of brome hay with DDGS decreased CH_4 production but replacement of corn with DDGS increased CH_4 production. As the supply of DDGS will increase with increased corn-ethanol production it is likely that DDGS will

be more frequently considered as a replacement for current dietary components. We conclude that the impact of feeding DDGS on CH_4 emission from ruminants will be desirable to the extent that this corn-ethanol production coproduct replaces forage rather than grain.

Table 3.1. Composition of diet offered to ruminal fluid donor heifer (DM basis)

Ingredient	%
Brome hay	69.70
Dry rolled corn	14.86
Soybean meal	15.04
Sodium chloride	0.32
Trace mineral premix[1]	0.05
Vitamin A, D, and E premix[2]	0.03

[1]Premix contained 6% Zn, 5% Fe, 4% Mn, 2% Cu, 2,000 ppm I, 500 ppm Co.

[2]Premix contained 30,000 IU vitamin A, 6,000 IU vitamin D, 7.5 IU vitamin E per g.

Table 3.2. Nutrient content of feedstuffs (% on DM basis; in vitro experiments)

Item	DDGS	Brome hay	Fine ground corn
NDF	28.4	68.9	15.3
CP	29.3	14.6	10.6
Starch	8.1	1.1	71.6
EE	9.9	2.4	3.7

Table 3.3. Diet composition and nutrient content (% on DM basis; Experiment 1)

	BRAN	30DDGS	DDGS+BRAN
Ingredient			
Brome hay	56	60	30
Dried distillers grains	0	30	30
Corn bran	30	0	30
Molasses	5	5	5
Soybean meal	4	0	0
Sheep mineral mix[1]	2	2	2
Ammonium chloride	2	2	2
Salt	1	1	1
Nutrient Content			
NDF	61.9	48.6	50.5
CP	15.3	18.5	18.8
Starch	5.4	4.4	7.0
EE	2.4	5.1	5.2

[1]Mineral mix contained 15% Ca, 8% P, 18% NaCl, 12 ppm Se; and 132 IU vitamin A, 64 IU vitamin D, 0.7 IU vitamin E per g.

Table 3.4. Least squares means ± SEM indicating the effect of replacing brome hay or corn bran with distillers dried grains plus solubles (DDGS) on intake, digestibility, methane (CH_4) emissions, and ruminal fluid properties

Item	BRAN	30DDGS	DDGS+BRAN	SEM	*P*-value[1]
DMI (kg)	0.252	0.255	0.253	0.015	0.80
DM Digestibility (%)	62.9[a]	63.1[a]	68.6[b]	0.8	<0.01
CH_4 (mmol/h)	29.4[a]	25.6[b]	24.8[b]	2.6	0.03
CH_4 (mmol•h^{-1}•kg^{-1})[2]	158[a]	135[b]	113[c]	9	<0.01
pH	6.20	6.26	6.18	0.09	0.49
Acetate (mM)	33.3	26.6	28.9	1.8	0.08
Propionate (mM)	11.3[a]	9.0[b]	10.2[c]	0.3	<0.01
Butyrate (mM)	4.03	3.51	3.69	0.29	0.42

[abc]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1] *F*-test statistic for the effect of diet.

[2]CH_4 production rate per kg of digested DM.

Table 3.5. Diet composition and nutrient content (% on DM basis; Experiment 2)

	CORN	CORN/DDGS
Ingredient		
Fine ground corn	71.4	43.7
DDGS	0.0	29.9
Alfalfa	10.0	10.0
Brome hay	10.0	10.0
Sheep mineral mix[1]	2.5	2.5
Ammonium chloride	2.5	2.5
Corn oil	2.2	0.0
Lignin sulfonate	1.4	1.4
Nutrient Content		
Starch	56.6	44.6
NDF	18.4	25.9
CP	14.3	18.5
EE	4.0	6.1

[1]Mineral mix contained 15% Ca, 8% P, 18% NaCl, 12 ppm Se; and 132 IU vitamin A, 64 IU vitamin D, 0.7 IU vitamin E per g.

Table 3.6. Least squares means ± SEM indicating the effect of replacing corn with distillers dried grains plus solubles (DDGS) on intake, digestibility, methane (CH_4) emissions, and ruminal fluid properties

Item	CORN	CORN/DDGS	SEM	*P*-value[1]
DMI (kg)	0.607	0.581	0.038	<0.01
DM Digestibility (%)	78.1	75.8	1.3	0.15
CH_4 (mmol/h)	33.0	39.2	7.1	0.12
CH_4 (mmol•h^{-1}•kg^{-1})[2]	68.3	88.9	13	0.01
pH	5.85	5.49	0.14	0.06
Acetate (mM)	24.3	24.3	2.4	0.99
Propionate (mM)	17.0	14.3	2.1	0.37
Butyrate (mM)	6.79	6.12	1.33	0.69

[1] *F*-test statistic for the effect of diet.

[2]CH_4 production rate per kg of digested DM.

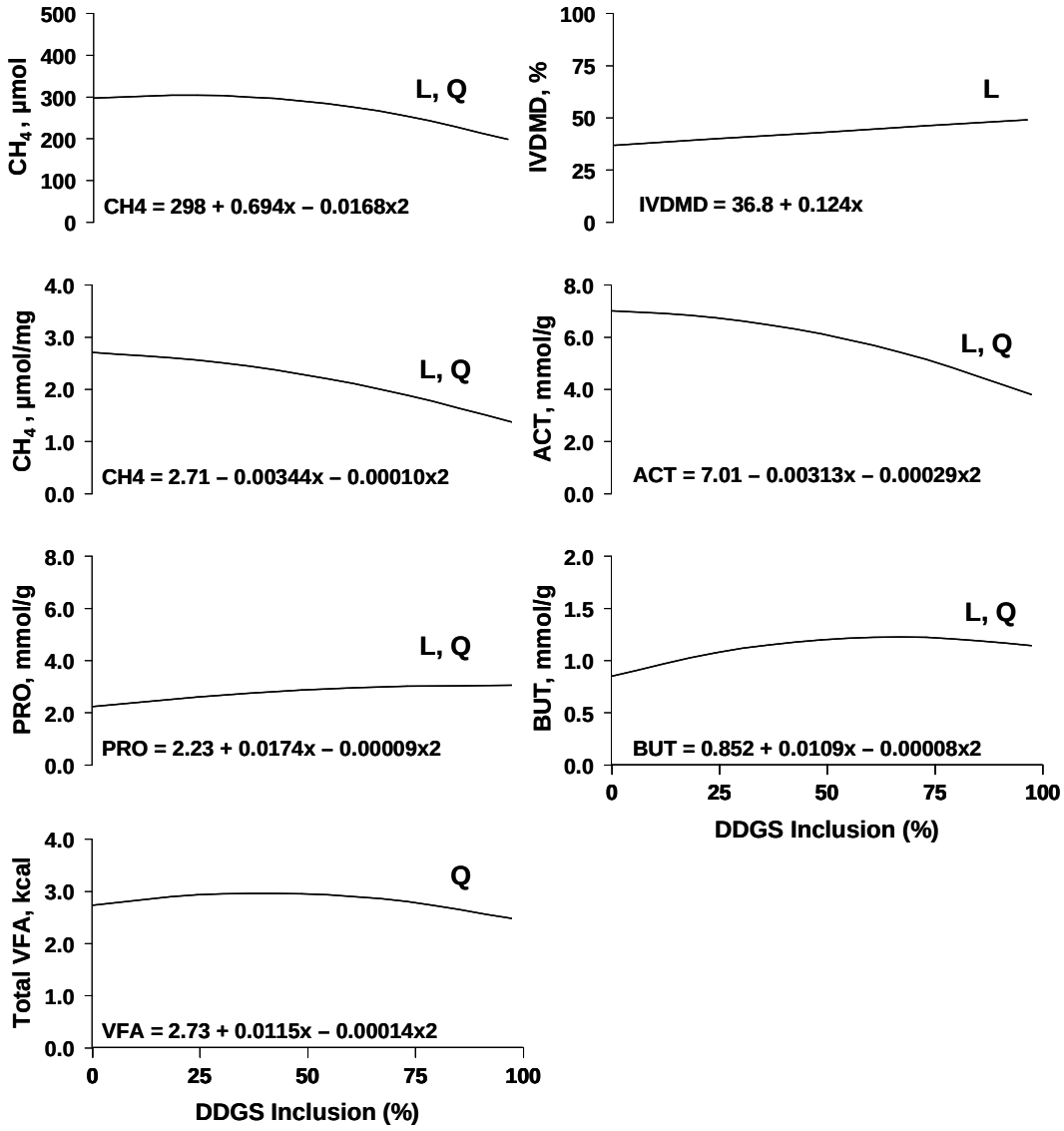


Figure 3.1. Response curves for total methane (CH_4) production, IVDMD, CH_4 production per milligram of digestible DM, acetate (ACT) production, propionate (PRO) production, butyrate (BUT) production, and total kilocalories produced in the form of VFA when dried distillers grains plus solubles (DDGS) replaced brome hay as a substrate for in vitro fermentation. L = linear coefficient was significant ($P < 0.05$), Q = quadratic coefficient was significant ($P < 0.05$).

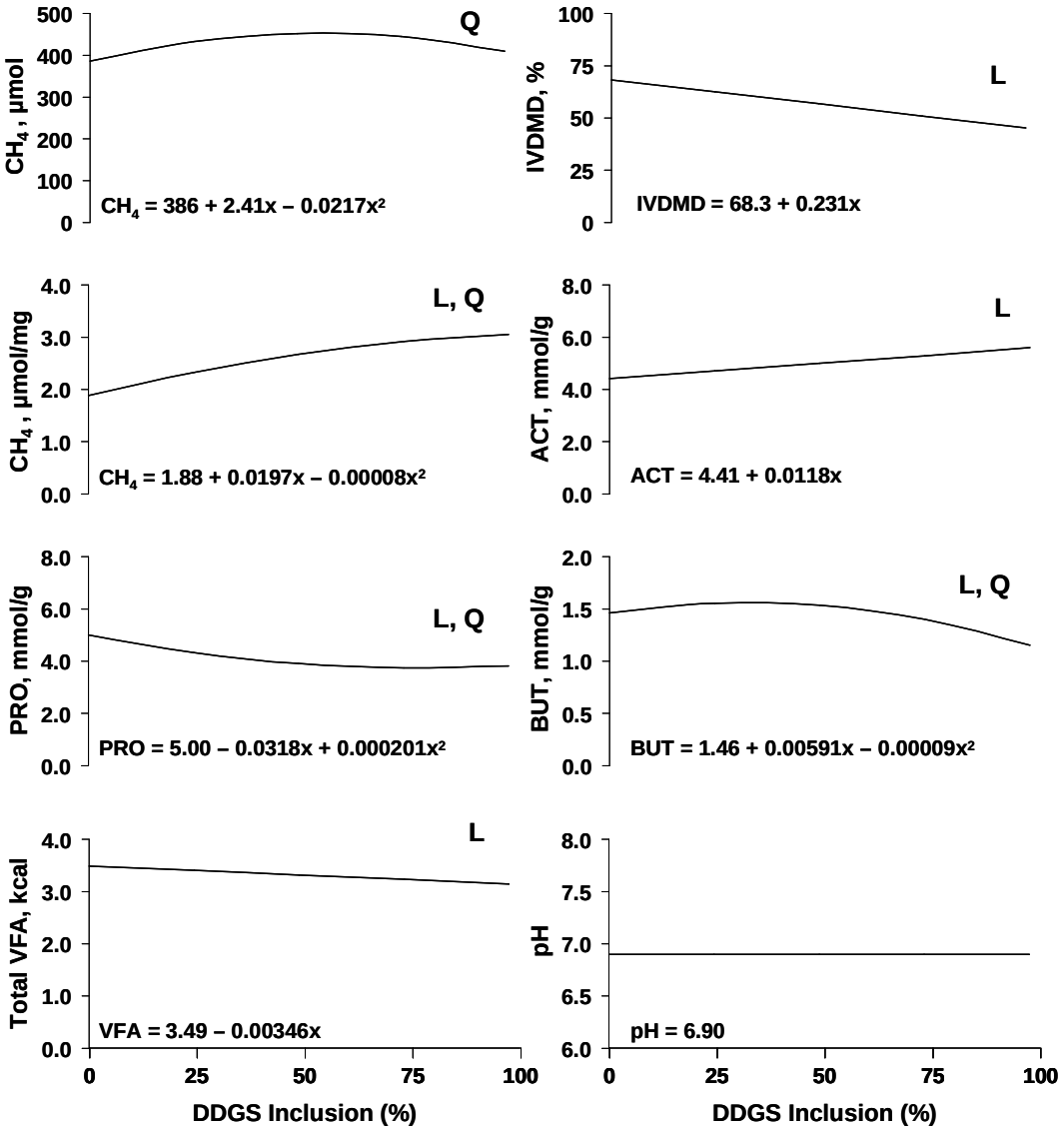


Figure 3.2. Response curves for total methane (CH_4) production, IVDMD, CH_4 production per milligram of digestible DM, acetate (ACT) production, propionate (PRO) production, butyrate (BUT) production, total kilocalories produced in the form of VFA, and final culture pH when dried distillers grains plus solubles (DDGS) replaced corn as a substrate for in vitro fermentation. L = linear coefficient was significant ($P < 0.05$), Q = quadratic coefficient was significant ($P < 0.05$).

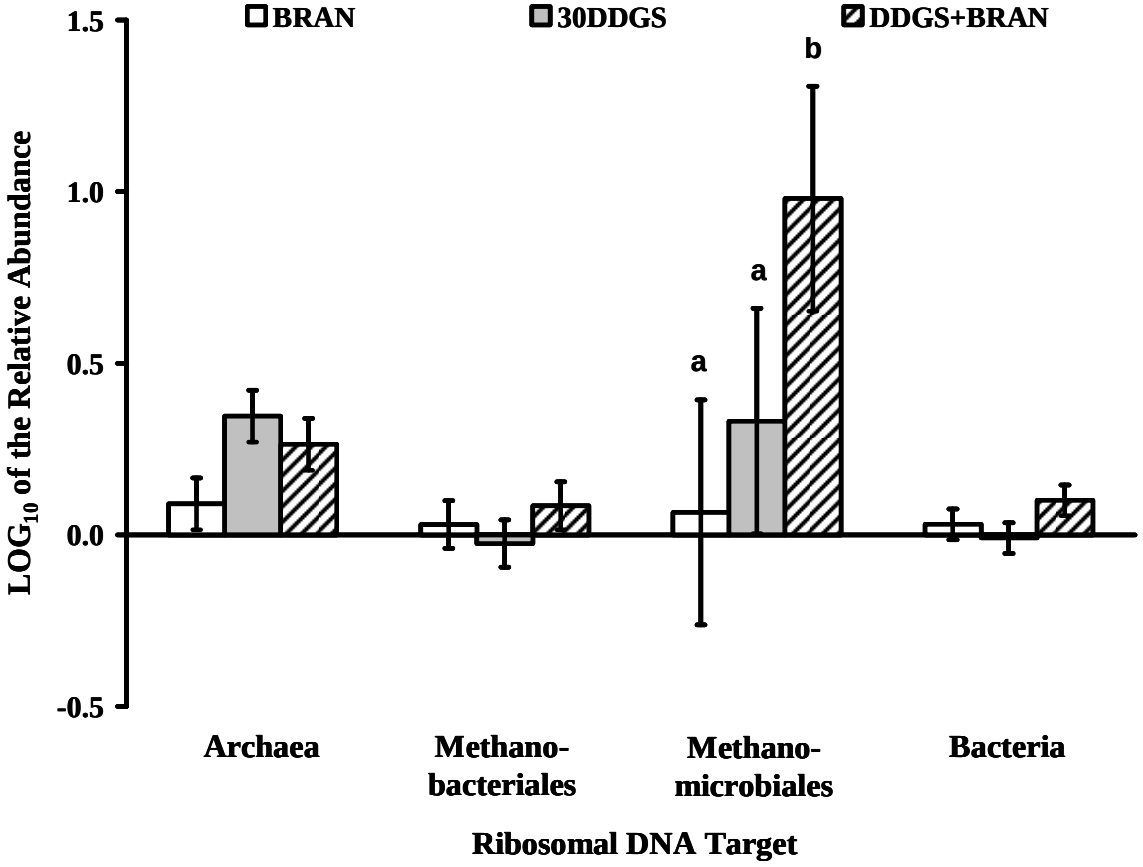


Figure 3.3. Effect of brome hay-based rations containing 30% corn bran (BRAN), 30% DDGS (30DDGS), or 30% DDGS and 30% corn bran (DDGS+BRAN) on the abundance of ruminal Archaea, Methanobacteriales, Methanomicrobiales, and Bacteria. Individual bars represent least squares means with SEM. Within a ribosomal DNA target, means lacking a common superscript differ ($P < 0.05$). *P*-values for the main effect of treatment: Archaea ($P = 0.06$), Methanobacteriales ($P = 0.44$), Methanomicrobiales ($P = 0.02$), and Bacteria ($P = 0.16$).

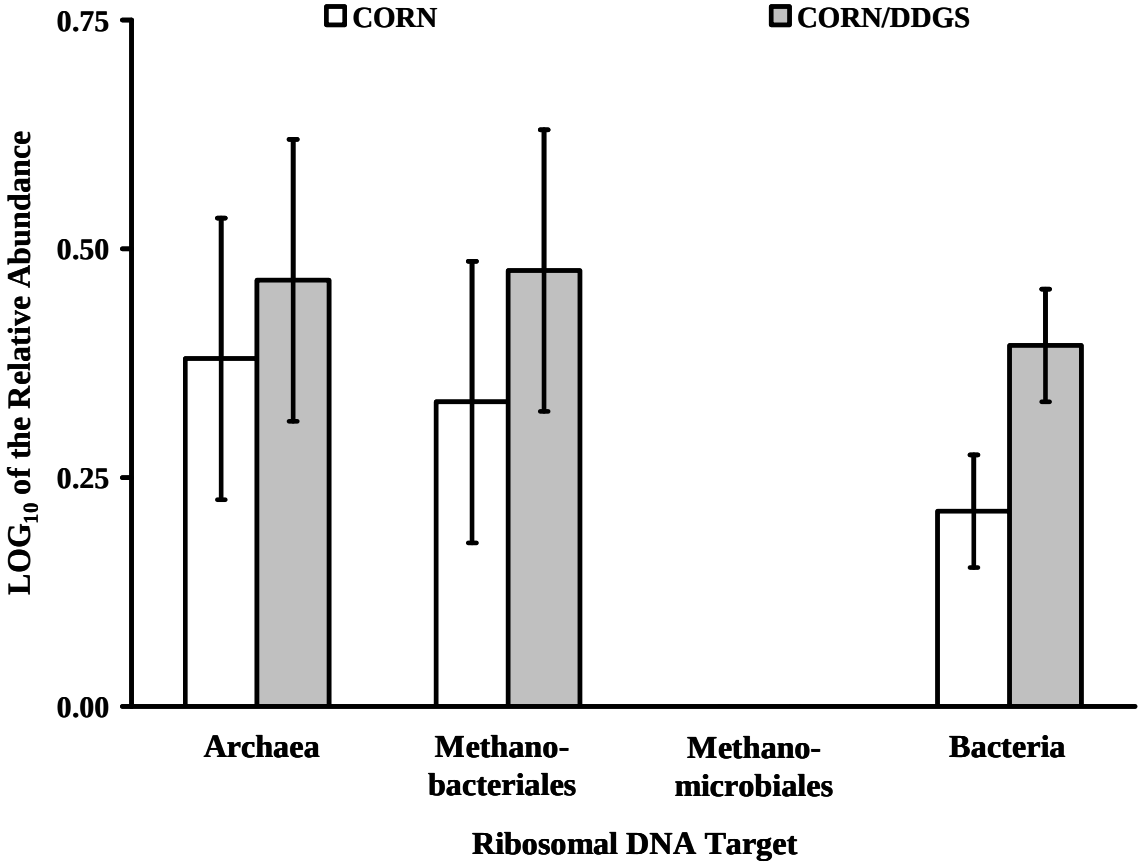


Figure 3.4. Effect of rations containing 71% corn and 2.2% corn oil (CORN), or one in which DDGS replaced corn for 30% of the diet (CORN/DDGS) on the abundance of ruminal Archaea, Methanobacteriales, Methanomicrobiales, and Bacteria. Individual bars represent least squares means with SEM. *P*-values for the main effect of treatment: Archaea (*P* = 0.61), Methanobacteriales (*P* = 0.43), and Bacteria (*P* = 0.06).

CHAPTER IV

Effect of dietary carbohydrate source and 2-bromoethanesulfonate on ruminal methanogen populations as assessed by real-time PCR

ABSTRACT: Our objectives were to determine if a relationship exists between ruminal methane (CH_4) emissions and ribosomal DNA indicators of methanogens, and to determine how the abundance of ruminal methanogens is affected by dietary carbohydrate and 2-bromoethansulfonate (BES) treatment. Ruminally fistulated (n = 3) lambs were offered a ration (2.75% BW) containing 100% brome hay (BROME), or one in which corn replaced brome hay for 33% (MIXED) or 70% (CORN) of the ration. As lambs were maintained on the CORN ration, BES (2 g/d) was infused (day 0) intraruminally at 0800 and 1600 for 7 d. Following adaptation to each diet, and on selected days of the BES experiment, exhaled gas was collected to determine CH_4 production by the SF_6 tracer technique. Feces and orts were collected to determine digestibility, and ruminal fluid was collected for determination of pH and VFA concentrations. Real-time PCR was used to monitor the changes in ruminal microbe populations. The BROME diet resulted in a greater ($P = 0.02$) amount of CH_4 emitted per kilogram of DM compared to the CORN diet (207 vs. 121 mmol $\bullet h^{-1} \bullet kg^{-1}$, respectively). At 1200, 1600, and 2000 the BROME diet resulted in a greater ($P < 0.05$) relative abundance of total Archaea compared to the CORN diet. Compared to both the MIXED and CORN diets, the BROME diet resulted in a lesser ($P < 0.02$) relative abundance of Methanobacteriales but a greater ($P < 0.07$) relative abundance of Methanomicrobiales. Relative to pretreatment values, CH_4 emissions per kilogram of

DM were reduced ($P < 0.05$) by BES an average of 84% on days 0, 1, and 6. The relative abundance of Archaea and Methanobacteriales was reduced ($P < 0.01$) on days 3 and 6 when compared to those of days -1, 0, and 1. We conclude that ribosomal DNA indicators of methanogens may be used as indicators of ruminal CH_4 emissions and that frequent BES treatment may be an effective strategy for reducing CH_4 emissions when ruminant animal consume concentrate-based diets.

INTRODUCTION

Ruminant animal methane (**CH_4**) production should be targeted for inhibition because: 1) it represents a significant loss of feed GE (Johnson and Johnson, 1995) and 2) CH_4 is a greenhouse gas and enteric fermentation is the second largest source from anthropogenic activities in the United States (EPA, 2007). One strategy that attenuates ruminal methanogenesis is the replacement of dietary structural carbohydrates with nonstructural carbohydrates (Johnson and Johnson, 1995; Johnson et al., 2000; Boadi et al., 2004). Also, the compound 2-bromoethansulfonate (**BES**) is a potent inhibitor of CH_4 production by ruminal microorganisms both in vitro (Martin and Macy, 1985; Nollet et al., 1997; Ungerfeld et al., 2004) and in vivo (Immig et al., 1996). However, ruminal microflora quickly (within 3 d following treatment) become resistant to the effects of BES and methanogenesis resumes (Immig et al., 1996). In vitro data indicate that individual species of methanogens differ in their sensitivity to BES (Ungerfeld et al., 2004). We would like to know how the ruminal methanogen population is affected by dietary change and by BES.

The use of molecular techniques to study the rumen microbial ecosystem has revealed that the methanogenic population is more diverse (Whitford et al., 2001; Nicholson et al., 2007; Wright et al., 2007) than previously believed when knowledge was based on culture-base techniques (Joblin, 2005). Real-time PCR assays can be used for the detection of specific ruminal microbes (Denman and McSweeney, 2005), and domain- and order-specific probes have been validated for the detection of methanogens (Yu et al., 2005).

We hypothesized that replacing dietary forage with grain and treatment with BES would reduce the abundance of Archaea in the rumen. We also hypothesized that the acquisition of resistance to BES would be characterized by the proliferation of an individual order of methanogenic Archaea.

MATERIALS AND METHODS

Sheep Experiments

Animal care. The procedures used for this experiment were approved by the University of Nebraska's Institutional Animal Care and Use Committee (protocol #05-07-046D).

Forage vs. concentrate experiment. The objectives were to: 1) determine if the replacement of dietary brome hay with corn would decrease the abundance of subsets of ruminal Archaea, and 2) determine if a relationship exists between ruminal CH_4 emissions and ribosomal DNA indicators of methanogens. Three ruminally cannulated crossbred lambs (30.4 ± 6.9 kg) were offered (2.75% BW at commencement of experiment) a diet containing 100% brome hay on days 0 through 9 (**BROME**), 67% brome hay and 33% dry rolled corn on days 19 through 24 (**MIXED**), and 30% brome

hay and 70% dry rolled corn on days 35 through 40 (**CORN**). Lambs were adapted to grain by replacing (3.33% per day) dietary brome hay with dry rolled corn on days 10 through 19 and days 25 through 35. Diets were offered twice daily at 0800 and 1600. Exhaled gas was collected from 0800 to 1400 and CH_4 production rate was determined on days 7 to 8, 22 to 23, and 38 to 39. On days 9, 24, and 40 ruminal fluid was collected at 0800, 1200, 1600, and 2000 using a suction strainer. Immediately upon collection the pH of the sample was recorded, and the samples were frozen and stored for later VFA analysis.

BES experiment. The objective was to determine which subset of Archaea allowed for the acquisition of resistance to chronic in vivo BES treatment. Following the completion of the forage vs. concentrate experiment, lambs were maintained on the CORN diet and on day 0 administered 1 g of BES twice daily (immediately prior to feeding) for 7 days. The BES was dissolved in 10 mL of water, which was infused directly into the rumen via an indwelling suction strainer and followed by 30 mL of water to purge the strainer. Exhaled gas was collected from 0800 to 1400 and CH_4 production rate was determined on days 0, 1, 3, and 6. Also on these days, ruminal fluid was collected at 0800 (prior to BES administration), 1200, 1600 (prior to BES administration), and 2000 utilizing the indwelling suction strainer. Immediately upon collection the pH of the sample was recorded, and the samples were frozen and stored for later VFA analysis.

Gas collection and analysis. Production rate of CH_4 was determined by procedures modified from those developed by Johnson et al. (1994). Brass permeation

tubes containing 135 to 512 mg of SF_6 and with known release rates (1,480 to 1,634 ng of SF_6/min) were placed in the rumen of lambs prior to the first gas collection period. On each collection day exhaled gas was drawn into evacuated (50 mmHg) PVC collection canisters for a 6-h period. Gas was drawn through 46 cm of capillary tubing (128 µm i.d.; Alltech Associates, Inc., Deerfield, Illinois) with an in-line 15-µm filter (Swagelok, Solon, Ohio) and nosepiece that rested above the animal's mouth and nose (Appendix II). Analysis of CH_4 was conducted utilizing a HP 5890 gas chromatograph (Agilent Technologies, Inc., Palo Alto, California) fit with a flame ionization detector and Propak Q 80/100 column (Restek, Bellefonte, Pennsylvania). Column, injector, and detector temperatures were 60, 100, and 150ºC, respectively and N_2 (20 mL/min) was used as the carrier gas. Analysis of SF_6 was conducted utilizing a HP 6890 gas chromatograph (Agilent Technologies, Inc.) fit with a micro-electron capture detector and HP-PLOT Molesieve 5A column (Agilent Technologies, Inc.). Column, injector, and detector temperatures were 40, 35, and 250ºC, respectively, and N_2 (1 mL/min) was used as the carrier gas.

Feedstuff and VFA Analyses

Feedstuff analyses. The DM concentration of diet samples, orts, and feces was determined by drying samples in a 60ºC oven for a minimum of 48 h. The NDF content of diet samples was determined according to the procedure of Van Soest et al. (1991) with the filter bag technique using the ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, New York) following a pre-extraction with acetone, and the inclusion of sodium sulfite and heat stable alpha-amylase. The CP content (%N × 6.25)

of diet samples was determined using a combustion-method N analyzer (Leco FP-528, Leco Corp., St. Joseph, Missouri) according to method No. 990.03 (AOAC, 1995). Starch and EE concentrations were analyzed using methods No. 996.11 and No. 920.39 (AOAC, 1995), respectively.

VFA analysis. The concentration of VFA was determined as previously described (Erwin et al., 1961) utilizing a HP 5890 gas chromatograph (Agilent Technologies, Inc.) fit with a Supelco 12144 column.

Relative Quantification of Microbes

DNA extraction. Total genomic DNA was extracted from fresh ruminal fluid that had been strained through 4 layers of cheesecloth utilizing the RBB+C method (Yu and Morrison, 2004), which employs a QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, California) following repeated bead beating steps to disrupt cell membranes. The quality and quantity of DNA samples were determined with gel electrophoresis and by spectrophotometry, respectively.

Real-time PCR. The relative presence of targets for 2 domain-specific (Bacteria and Archaea) and 4 order-specific (Methanobacteriales, Methanomicrobiales, Methanococcales, and Methanosarcinales) primer and probe sets (Yu et al., 2005) was determined for each sample in duplicate with real-time PCR utilizing an AB7700 (Applied Biosystems, Foster City, California). Each reaction well contained 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 4.2 µL of community DNA, 1.25 µL of the forward and reverse primers (final concentration, 500 nM), 0.4 µL of the corresponding probe (final concentration, 150 nM), and nuclease-free water to a final

volume of 25 µL. The following reaction conditions were applied to each well: an initial 2-min incubation at 50ºC; a 10-min incubation at 95ºC; and 45 cycles of denaturation at 95ºC for 10 s, annealing at 55ºC for 30 s, and extending at 60ºC for 1 min.

An optimal in-well DNA concentration was established for each primer and probe set to maximize the DNA present in each well and allow for the detection of targets present in small amounts while avoiding the inhibitory effects associated with most DNA samples (Gallup and Ackermann, 2006). The threshold cycle (**Ct**) values for each primer and probe set were determined with a test plate containing serially diluted samples from a pooled stock. This stock was created by combining aliquots of each experimental sample. The Ct values were then plotted against the $LOG_{(10)}$ of the dilution factor, and the linear portion of this plot indicated the concentrations of DNA at which inhibition was absent. The DNA concentration used for each primer and probe set was 60% less than the maximum determined to avoid inhibition of the PCR reaction. The optimal in-well DNA concentrations for the primer and probe sets targeting Archaea, Methanobacteriales, Methanomicrobiales, and Bacteria were 1.396, 0.558, 0.265, and 0.089 ng/µL, respectively. Four samples containing decreasing concentrations of DNA and diluted from the stock solution were tested in duplicate on each PCR plate. The difference between the average Ct value of these standards on all plates and the average Ct value for the 4 standards on each plate was added to the Ct of all samples on each respective plate to neutralize inter-plate variation. The Ct of each plate's four standards was plotted on the $LOG_{(10)}$ of the dilution factor. The slope of this line indicated the

efficiency of amplification ($\mathbf{E_{AMP}}$). The Ct value for each unknown was then standardized to a common denominator with equation 2.1.

Calculations

CH_4 emmissions. Emission rates of CH_4 were calculated by multiplying the permeation rate of SF_6 by the ratio of the concentrations of CH_4 and SF_6 present in the collections canisters (Johnson et al., 1994). Emission rates of CH_4 per kilogram of digested DM were calculated by dividing the CH_4 emission value by the product of the amount of DM consumed on the day of collection multiplied by the mean DM digestibility for the diet.

Real-time PCR. For each primer and probe set, the average Ct value acquired at 0800 during the period the animals were offered the BROME diet (forage vs. concentrate experiment) or at 0800 on day -1 (BES experiment) was used as a "Reference Ct" and a value of 1.0 relative abundance units was assigned to these samples. The abundance of target for each primer and probe set for all other samples was calculated with the equation 2.2.

Statistical Analysis

All data were analyzed utilizing the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). Animal within day was the experimental unit. The model for the forage vs. concentrate experiment included the fixed effects of day, diet, and the day by diet interaction and the random effect of animal. The model for the BES experiment included the fixed effect of day and the random effect of animal. All of the real-time PCR data were $LOG_{(10)}$ transformed prior to analysis to account for non-constant variances as

indicated by the Shapiro-Wilk test for normality. Because VFA concentrations and the relative abundance of microbes were determined for samples collected from the same animals on an individual day, a repeated measures covariance structure was used. The AR(1) covariance structure was selected based on the Akaike information criterion. Least squares means were separated using the protected least significant difference test when a significant overall treatment *F*-test ($P < 0.05$) was detected. Methane production data from 1 animal on both days while being offered the MIXED diet were excluded from the analysis because the vacuum in that animal's collection canister failed to dissipate during the collection period. Ruminal fluid was unable to be collected because of a fouled suction strainer during 4, 3, 2, 1, and 4 individual times on different animals when they were offered the CORN diet (day -1 of the BES experiment), day 0, day 1, day 3, and day 6, respectively.

RESULTS AND DISCUSSION

Increasing the proportion of corn grain in diets offered to lambs from 0 to 70% did not affect total CH_4 emissions, but did decrease ($P = 0.02$) the amount of CH_4 produced per kilogram of digested DM (Table 4.1). One millimole of CH_4 has a heat of combustion of 0.2108 kcal (Owens and Goetsch, 1988). Therefore, during the 6-h collection period the BROME, MIXED, and CORN diets would have resulted in the loss of 81, 98, and 79 kcal, respectively, in the form of CH_4. Because corn contains a greater amount of DE compared to brome hay (3.84 vs. 2.43 Mcal/kg) (NRC, 1985) the replacement of corn with brome hay results in a lesser percentage of DE lost. The DM digestibility of the CORN diet was greater ($P < 0.01$) than that of either the MIXED or

BROME diets. Based on the nutrient composition of the brome hay and corn used (Table 4.2), the BROME diet contained the greatest amount of NDF but least amount of starch and ether extract. Alternately, the CORN diet contained the least amount of NDF but the greatest amount of starch and ether extract. The MIXED diet contained an amount of each of these three nutrients nearly equivalent to the mean of the BROME and CORN diets. Ruminal pH was not affected (Table 4.3). Compared to the BROME diet, the CORN diet resulted in greater acetate, propionate, and butyrate concentrations ($P = 0.05$, < 0.01, and $= 0.05$, respectively). Some caution is warranted when comparing VFA concentrations with CH_4 production. Concentration of VFA is a function of not only production rate, but also absorption and passage rates, and ruminal volume. Diet and time interacted ($P = 0.01$) to affect the relative abundance of ruminal Archaea (Figure 4.1). With the exception of 0800, the BROME diet resulted in a greater ($P < 0.05$) abundance of Archaea than did the CORN diet. Also, with the exception of 1600, the MIXED diet resulted in a greater ($P < 0.05$) abundance of Archaea than did the CORN diet. In comparison to both the MIXED and CORN diets, the BROME diet resulted in a lesser ($P < 0.02$) relative abundance of Methanobacteriales (Figure 4.2), but a greater ($P < 0.07$) relative abundance of Methanomicrobiales (Figure 4.3). The MIXED diet resulted in a lesser relative abundance of Bacteria than did the CORN diet (Figure 4.4).

Intraruminal administration of BES decreased the amount of CH_4 produced per kilogram of digested DM as indicated by a reduction ($P < 0.05$) on days 0 and 1 relative to pretreatment observations (Table 4.4). Ruminal methanogenesis on day 3 was not different from day -3, but on day 6 CH_4 production was suppressed ($P < 0.05$) compared

to pretreatment values. Dry matter intake, DM digestibility, pH, and VFA concentrations were not affected by BES treatment. The relative abundance of Archaea was decreased ($P < 0.01$) by day 3 and this suppression persisted to day 6 (Figure 4.5). In an identical fashion, the relative abundance of Methanobacteriales was lesser ($P < 0.01$) on days 3 and 6 relative to days -1, 0 and 1 (Figure 4.6). The relative abundance of Methanomicrobiales was not affected by treatment (Figure 4.7). On day 3 the relative abundance of Bacteria was suppressed ($P < 0.01$) at 0800 and 12004, but recovered by 2000 and were not different on day 6 (Figure 4.8).

Our first objectives were to: 1) determine if the replacement of dietary brome hay with corn would decrease the abundance of subsets of, or all, ruminal Archaea and 2) determine if a relationship exists between ruminal CH_4 emissions and ribosomal DNA indicators of methanogens. Assumptions made for interpretation of the real-time PCR include: 1) the efficiency of DNA extraction was similar for all samples and 2) ruminal volume was not affected by treatment. These are considered to be safe assumptions. As hypothesized, the replacement of brome hay with corn for 70% of the diet did decrease the relative abundance of Archaea and also decreased the amount of CH_4 produced per kilogram of digested DM. These results indicate that ruminal CH_4 production is positively correlated with ribosomal DNA indicators of total ruminal Archaea. Interestingly, the two orders of methanogens detected in our samples responded differentially. As the proportion of corn increased in the diet the relative abundance of Methanomicrobiales decreased, whereas that of Methanobacteriales increased. Such an increase likely indicates the compensatory or opportunistic proliferation of

Methanobacteriales in response to a niche created from the demise of the Methanomicrobiales population. The cause of a shift in the methanogenic population is not known but has been observed when animals consuming different diets are compared (Lin et al., 1997; Wright et al., 2007). We speculate that ruminal pH, bacterial population changes, or even the relative concentration of substrates utilized by the methanogens may be responsible for this observed change in the methanogenic population. Therefore, when selecting treatments with the intention of inhibiting methanogenesis, the diet and the effectiveness of that treatment against the respective dominant methanogens should be considered.

The objective of our second experiment was to determine which subset of Archaea allowed for the acquisition of resistance to chronic in vivo BES treatment. Surprisingly, after 6 days of treating with BES twice daily, the ruminal microflora did not acquire resistance to the inhibitory effects of treatment. The efficacy of BES for inhibiting ruminal methanogens has been well established in vitro (Martin and Macy, 1985; Nollet et al., 1997; Ungerfeld et al., 2004) and the ability of these microbes to acquire resistance has been reported to occur both in vitro (Immig et al., 1996; Ungerfeld et al., 2004) and in vivo (Immig et al., 1996). A possible explanation for the difference in the effect of in vivo BES treatment is that Immig et al. (1996) administered BES continuously (2 g/d), and we administered a pulse dose (1 g) of BES twice daily. Continuous exposure to BES likely results in the selective proliferation of resistant methanogens. However, after a pulse dose the concentration of inhibitor is continuously

decreasing and may allow for less resistant methanogens to flourish, and ultimately prevent the establishment of a resistant population.

The relative abundance of methanogens of the order Methanobacteriales mimicked that of total Archaea following BES treatment. This is likely because the abundance of methanogens of the order Methanomicrobiales was minimal at the commencement of the experiment, and Methanobacteriales was the dominant order of methanogens present. Methanomicrobiales was detected in 100% of samples from animals offered the BROME diet, 50% of the samples from animals offered the MIXED diet, and 33% of the samples from animals offered the CORN diet. Methanomicrobiales was detected in only 12% of the samples from animals offered the CORN diet and treated with BES. Ungerfeld et al. (2004) reported that *Methanomicrobium mobile* (of the order Methanomicrobiales) is resistant to the effects of BES and that *Methanobrevibacter ruminantium* (of the order Methanobacteriales) is not. Although the absence of a significant time effect on the relative abundance of Methanomicrobiales following BES treatment may indicate a lack of sensitivity to BES. This observation may also be a function of the scant relative abundance of Methanomicrobiales prior to treatment. Consequently, our data do not conclusively indicate the effectiveness of chronic in vivo BES treatment on methanogens of the order Methanomicrobiales. Nonetheless, we are confident to conclude that BES remained effective against methanogens of the order Methanobacteriales for 6 d.

We conclude that the drop in methanogenesis caused by feeding corn in place of brome hay is associated with a drop in the prevalence of Methanomicrobiales. In

addition, it also appears that frequent bolus treatment of BES may be an effective strategy for decreasing CH_4 emissions from ruminant animals consuming a concentrate-based diet.

Table 4.1. Least squares means indicating the effect of replacing corn with brome on intake, digestibility, and methane (CH_4) emissions

Item	BROME	MIXED	CORN	SEM	*P*-value[1]
DMI (kg)	0.675	0.776	0.733	0.028	0.07
DM Digestibility (%)	49.4[a]	60.0[b]	73.7[c]	3.1	<0.01
CH_4 (mmol/h)	64.2	77.6	62.5	27.6	0.18
CH_4 (mmol•h^{-1}•kg^{-1})[2]	207[a]	193[ab]	121[b]	57	0.05

[abc]Within a row, means without a common superscript letter differ (P < 0.05).

[1] *F*-test statistic for the effect of diet.

[2]CH_4 production rate per kg of digested DM.

Table 4.2. Nutrient composition of feedstuffs (% on DM basis; forage vs. concentrate experiment)

	Nutrient			
Ingredient	NDF	CP	Starch	EE
Brome hay	65	10.2	2.4	2.4
Dry rolled corn	14	9.5	70.4	4.6

Table 4.3. Least squares means indicating the effect of replacing corn with brome on ruminal fluid properties

	Diet				*P*-value[1]	
Item	BROME	MIXED	CORN	SEM	Diet	Diet × Time
pH	6.82	6.64	6.46	0.14	0.18	0.90
Acetate (mM)	48.4[a]	52.0[b]	50.8[b]	7.2	<0.01	<0.01
0800	36.3[a]	50.1[b]	44.3[c]			
1200	49.1	49.5	50.7			
1600	45.0[a]	51.6[b]	49.5[ab]			
2000	63.1[a]	56.7[b]	58.5[ab]			
Propionate (mM)	16.6[a]	18.8[b]	29.8[c]	1.8	<0.01	<0.01
0800	11.3[a]	15.5[b]	20.7[c]			
1200	17.7[a]	18.2[a]	29.6[b]			
1600	14.2[a]	18.8[b]	30.1[c]			
2000	23.3[a]	22.6[a]	38.9[b]			
Butyrate (mM)	5.68[a]	8.60[b]	8.00[ab]	0.14	0.03	0.06

[abc]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1] *F*-test statistic for the effect of Diet and the Diet × Time interaction.

Table 4.4. Least squares means indicating the effect of intraruminal BES treatment on intake, digestibility, methane (CH_4) emissions, and ruminal fluid properties

	Day											
Item	-3	-2	-1	0[1]	1	2	3	4	5	6	SEM	P-value[2]
DMI (kg)	0.676	0.683	0.652	0.711	0.771	0.788	0.688	0.734	0.722	0.795	0.048	0.22
DM Digestibility (%)		73.7					75.2				3.0	0.33
CH_4 (mmol/h)	57.0	67.9		12.3	12.3		21.0			12.1	17.5	0.08
CH_4 (mmol•h^{-1}•kg^{-1})[3]	109.6^{ab}	132.2^{a}		20.9^{c}	18.6^{c}		35.3^{bc}			18.4^{c}	30.6	0.04
pH			6.47	6.01	5.89		6.20			6.02	0.26	0.19
Acetate (mM)			49.4	38.7	50.2		42.1			45.3	10.2	0.77
Propionate (mM)			25.7	28.9	23.7		22.7			28.6	3.6	0.12
Butyrate (mM)			8.7	10.1	11.9		10.6			10.0	2.1	0.78

[abc]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1]BES administration commenced.

[2] *F*-test statistic for the effect of day.

[3]CH_4 production rate per kg of digested DM.

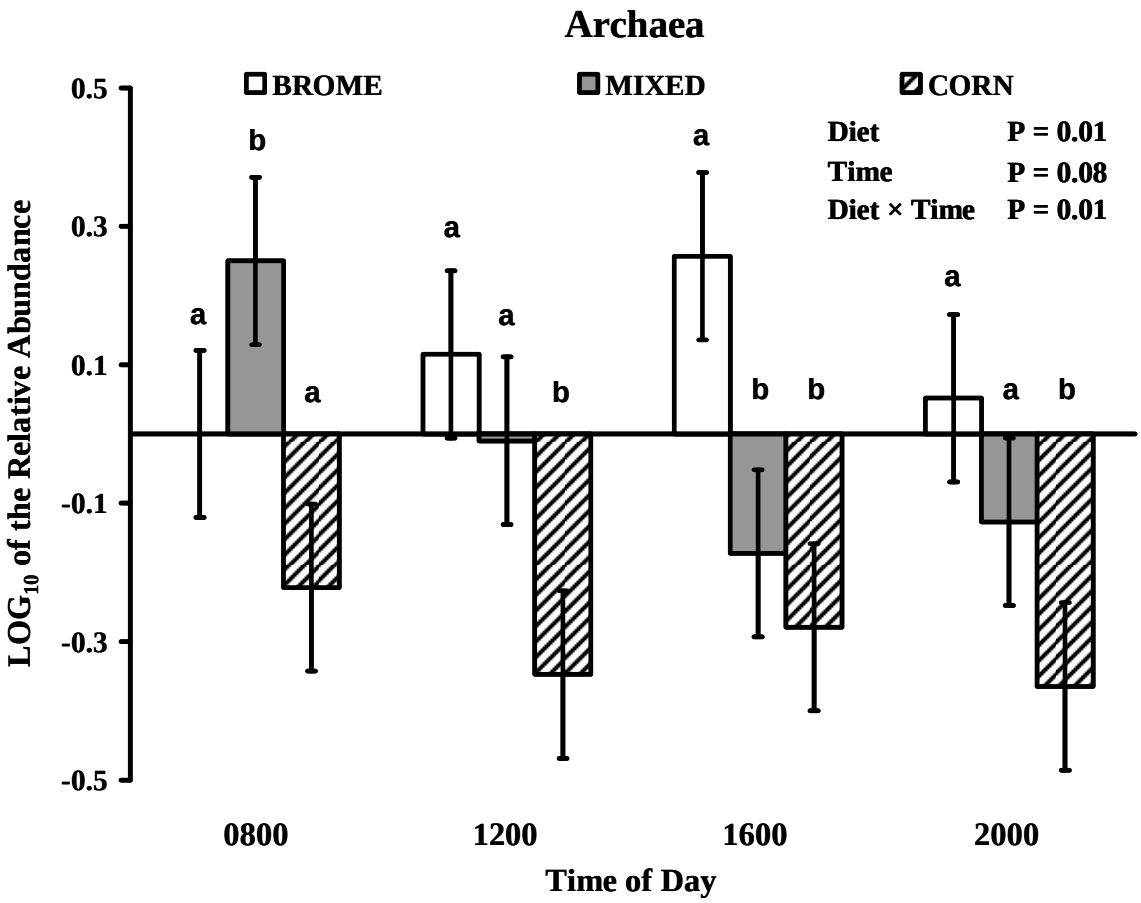


Figure 4.1. Effect of diets containing 100% brome hay (BROME), and those in which dry rolled corn replaced brome hay for 33% (MIXED) or 70% (CORN), on the abundance of ruminal Archaea. Individual bars represent least squares means with SEM. Within a time of day, means lacking a common superscript differ (*P* < 0.05).

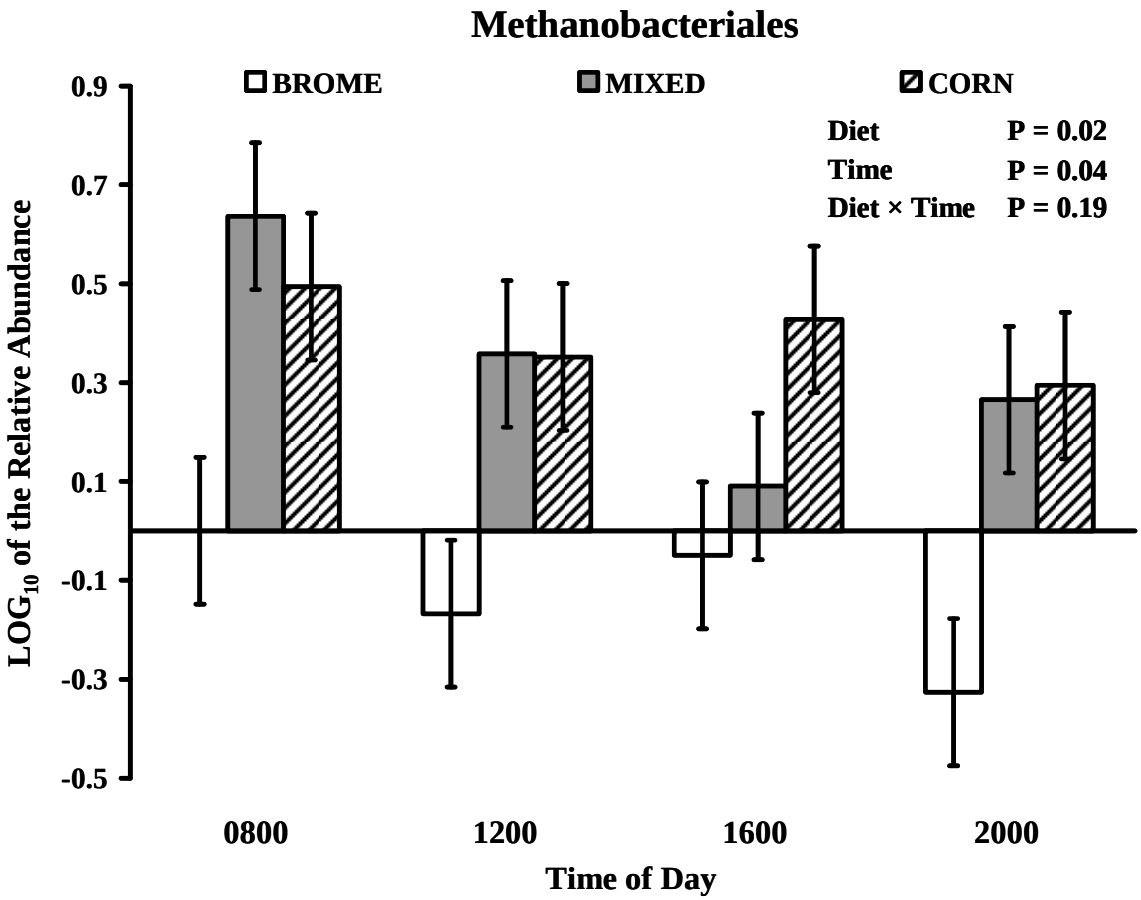


Figure 4.2. Effect of diets containing 100% brome hay (BROME), and those in which dry rolled corn replaced brome hay for 33% (MIXED) or 70% (CORN), on the abundance of ruminal Archaea of the order Methanobacteriales. Individual bars represent least squares means with SEM. Significant diet contrasts: BROME vs. MIXED (P = 0.02) and BROME vs. CORN (*P* = 0.01).

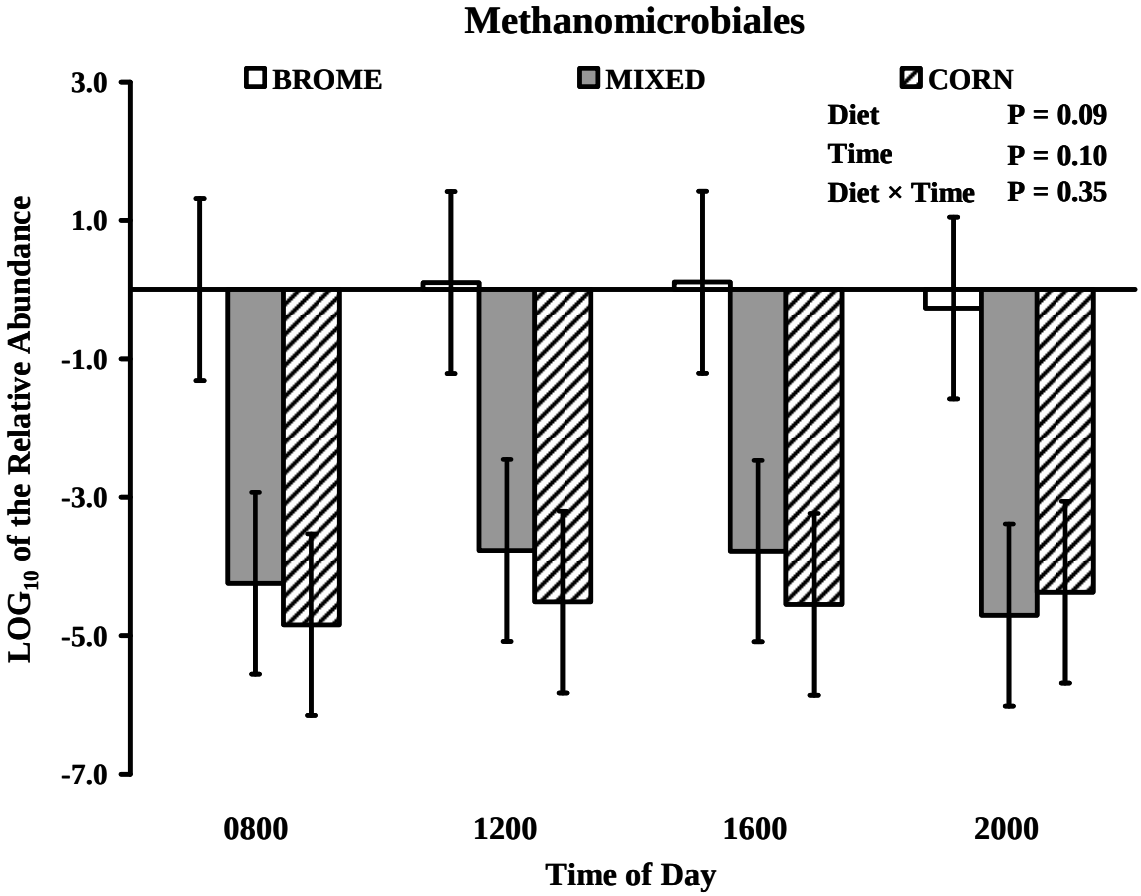


Figure 4.3. Effect of diets containing 100% brome hay (BROME), and those in which dry rolled corn replaced brome hay for 33% (MIXED) or 70% (CORN), on the abundance of ruminal Archaea of the order Methanomicrobiales. Individual bars represent least squares means with SEM. Significant diet contrasts: BROME vs. MIXED (P = 0.07) and BROME vs. CORN (*P* = 0.05).

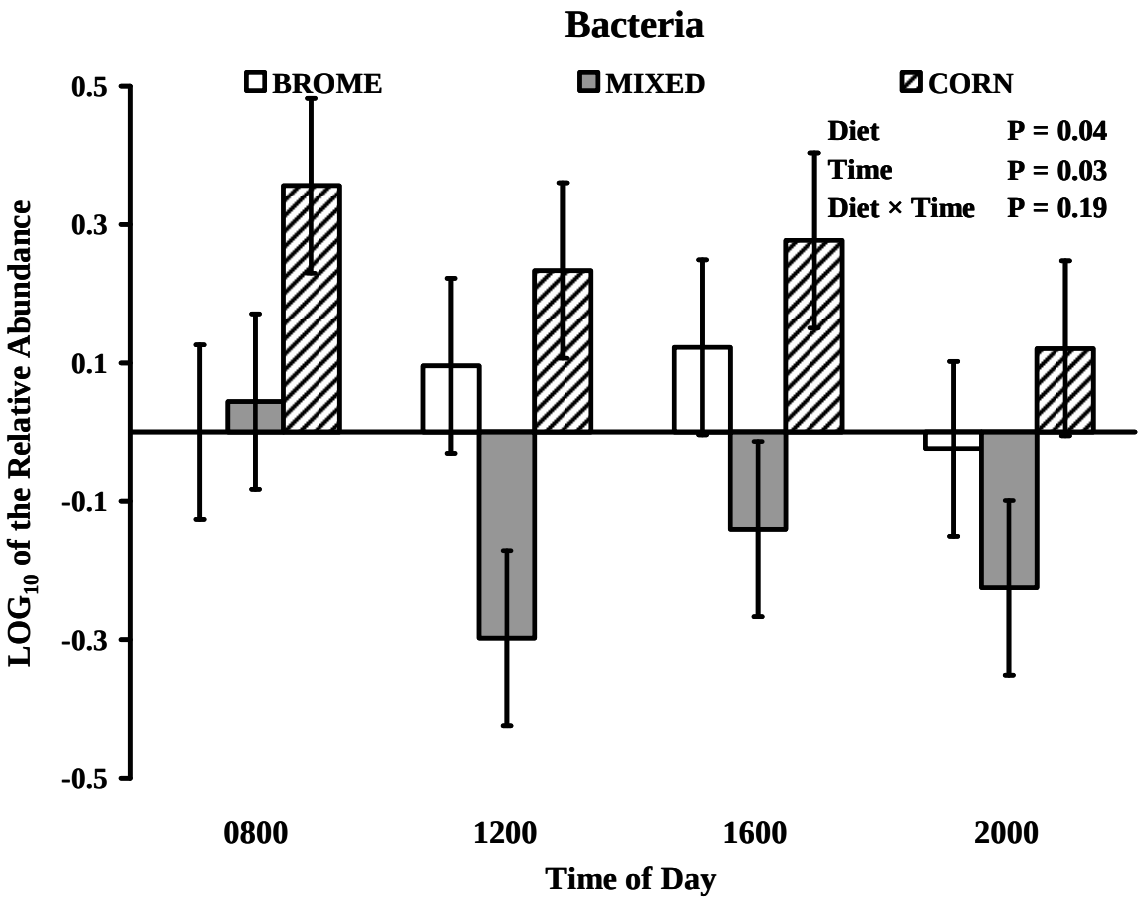


Figure 4.4. Effect of diets containing 100% brome hay (BROME), and those in which dry rolled corn replaced brome hay for 33% (MIXED) or 70% (CORN), on the abundance of ruminal Bacteria. Individual bars represent least squares means with SEM. Significant diet contrast: MIXED vs. CORN (*P* = 0.01).

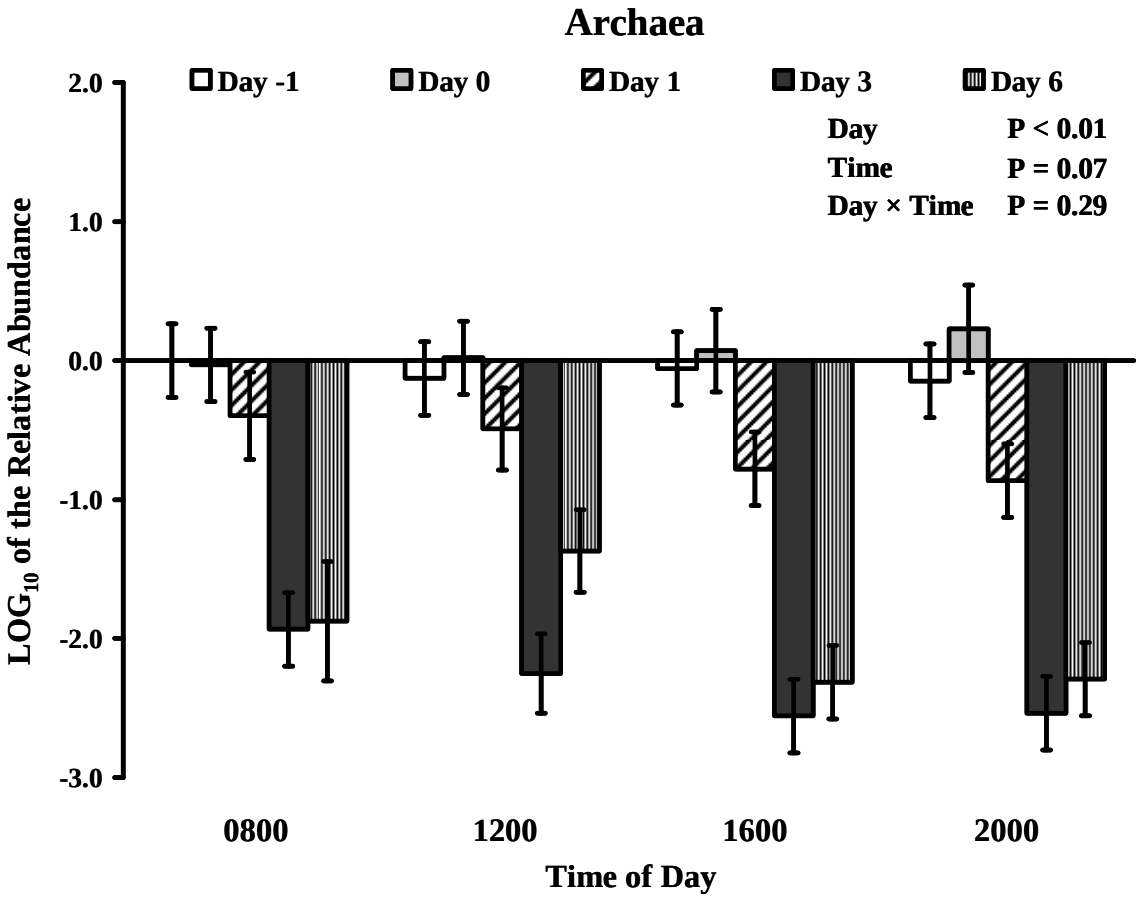


Figure 4.5. Effect of intraruminal administration (Day 0) of 2-bromoethansulfonate (BES) on the abundance of ruminal Archaea. Individual bars represent least squares means with SEM. Significant day contrasts: -1 vs. 3 ($P < 0.01$), -1 vs. 6 ($P < 0.01$), 0 vs. 1 ($P = 0.06$), 0 vs. 3 ($P < 0.01$), 0 vs. 6 ($P < 0.01$), 1 vs. 3 ($P < 0.01$), and 1 vs. 6 ($P < 0.01$).

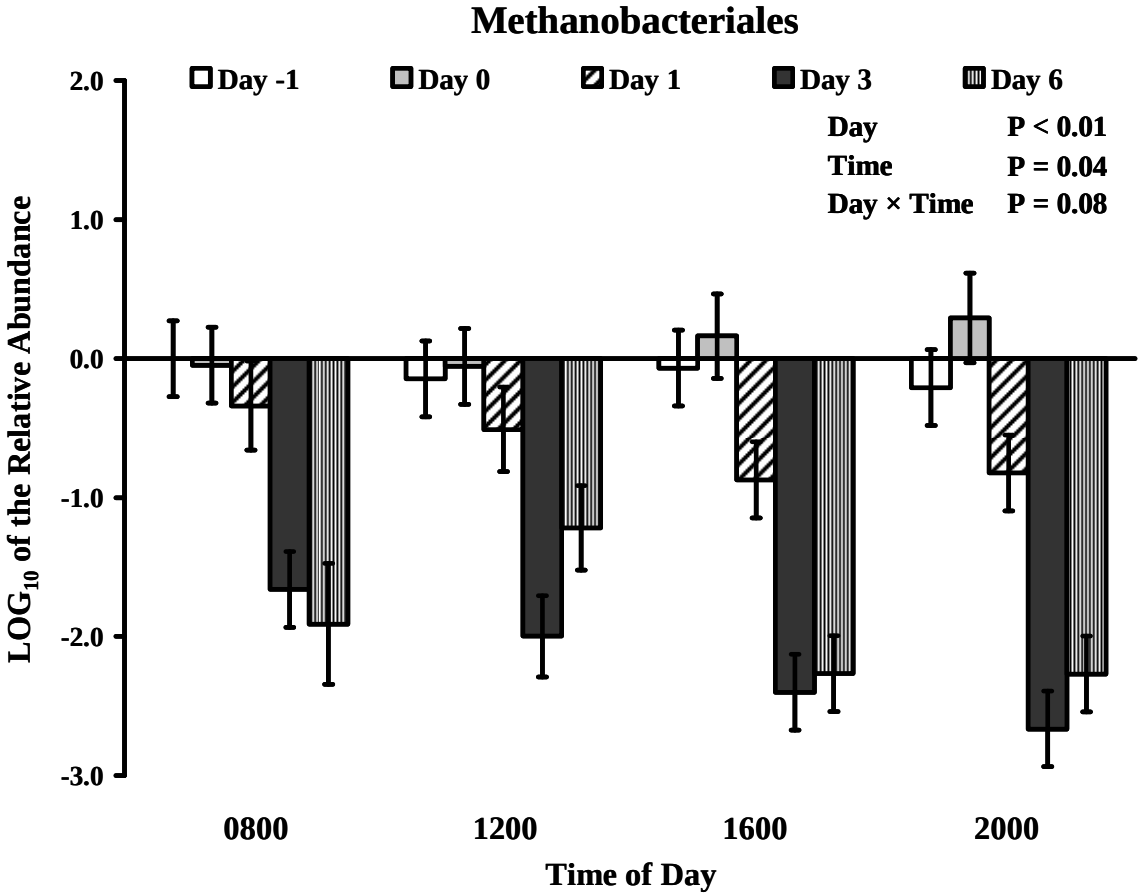


Figure 4.6. Effect of intraruminal administration (Day 0) of 2-bromoethansulfonate (BES) on the abundance of ruminal Archaea of the order Methanobacteriales. Individual bars represent least squares means with SEM. Significant day contrasts: -1 vs. 3 ($P < 0.01$), -1 vs. 6 ($P < 0.01$), 0 vs. 1 ($P = 0.06$), 0 vs. 3 ($P < 0.01$), 0 vs. 6 ($P < 0.01$), 1 vs. 3 ($P < 0.01$), and 1 vs. 6 ($P < 0.01$).

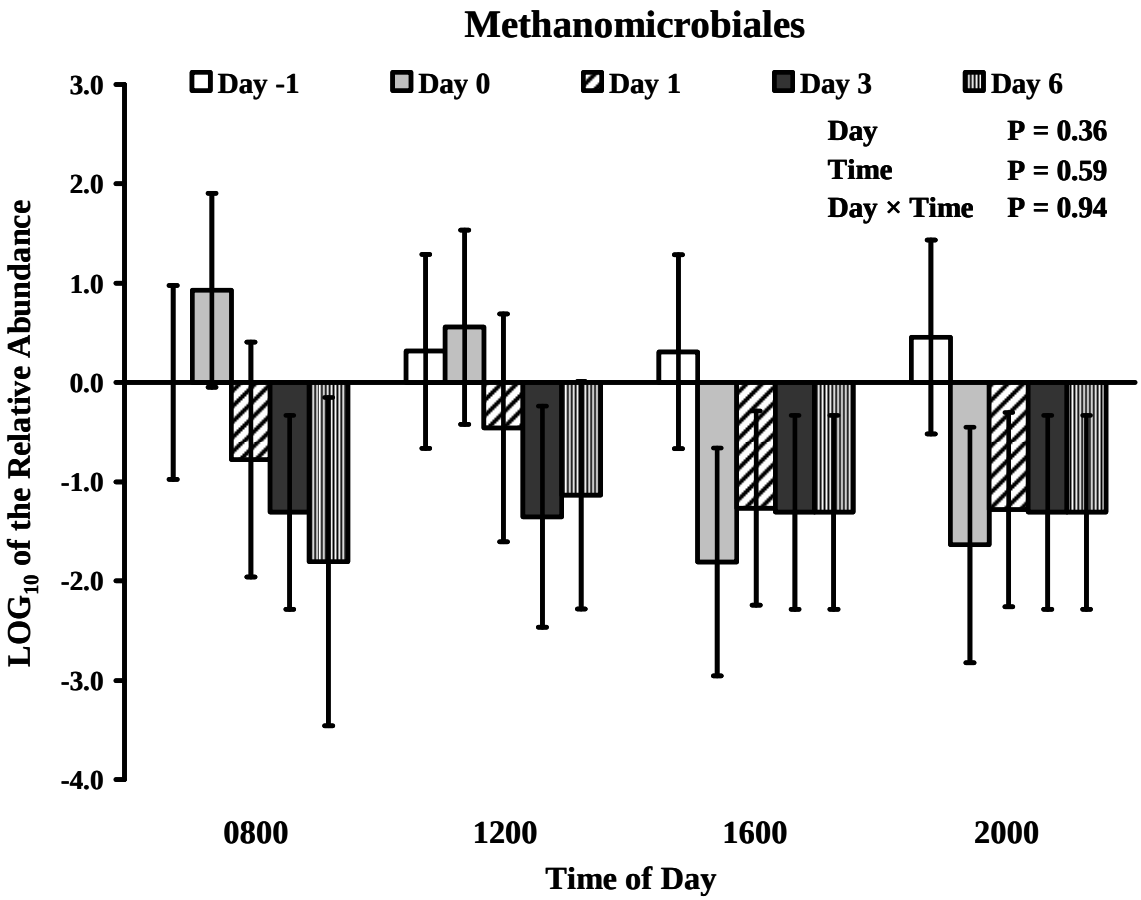


Figure 4.7. Effect of intraruminal administration (Day 0) of 2-bromoethansulfonate (BES) on the abundance of ruminal Archaea of the order Methanomicrobiales. Individual bars represent least squares means with SEM.

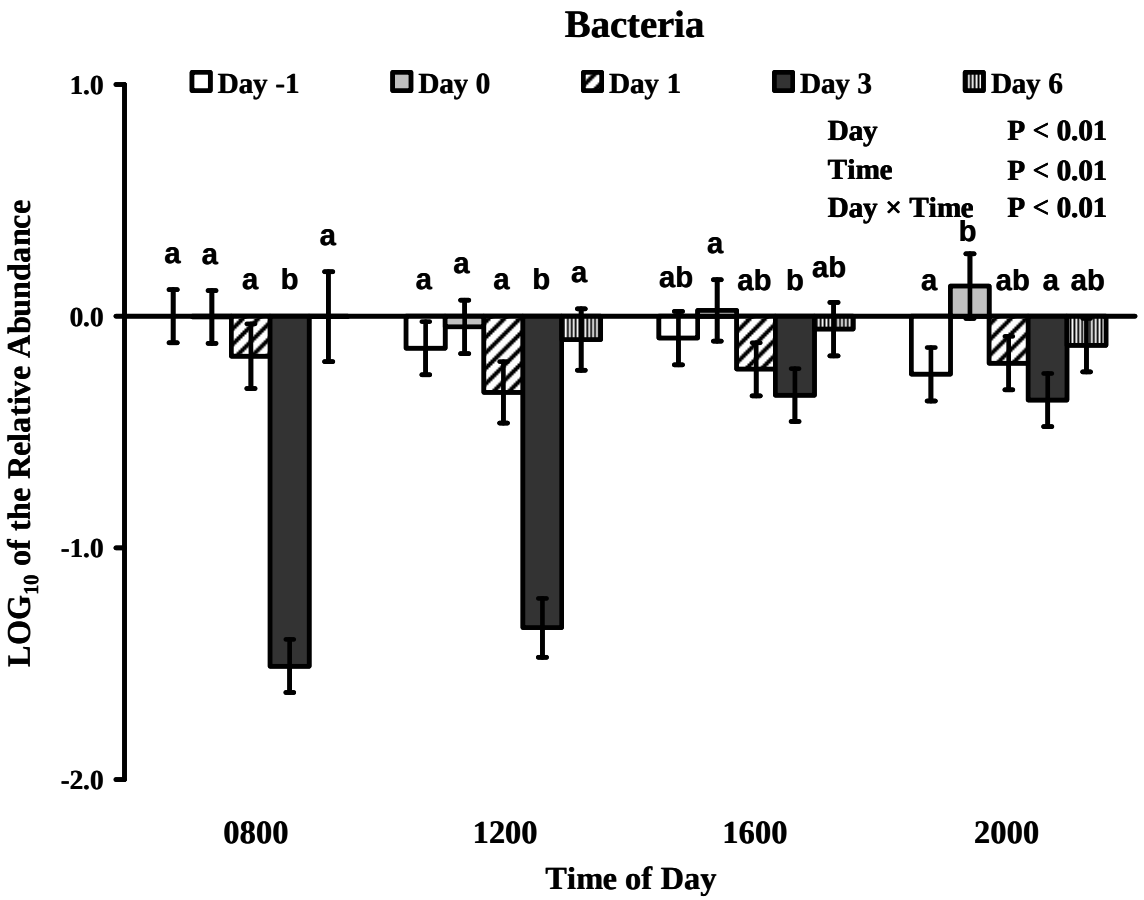


Figure 4.8. Effect of intraruminal administration (Day 0) of 2-bromoethansulfonate (BES) on the abundance of ruminal Bacteria. Individual bars represent least squares means with SEM. Within a time of day, means lacking a common superscript differ ($P < 0.05$).

CHAPTER V

Implications

Ruminal methane (CH_4) production may be reduced with dietary manipulations. In addition, in vitro data indicate that both structural analogs of para-aminobenzoate and *Yucca shidigera* extract are potent inhibitors of CH_4 production. The objectives of this work were to determine: 1) the extent to which ruminal cultures acquire resistance to a nitrofuranyl derivative of para-aminobenzoate (NFP) and an extract from the plant *Yucca shidigera* (Yucca); 2) the effect of distillers dried grains plus solubles (DDGS) on ruminal CH_4 production; 3) the effect of brome hay-based diets, corn-based diets, and in vivo 2-bromoethansulfonate treatment on ruminal CH_4 production; and 4) the effect of the above treatments on the methanogen population.

In terms of chronically inhibiting methanogenesis, the ability of ruminal microorganisms to acquire resistance to anti-methanogenic compounds has historically been the downfall of potential inhibitors of methanogenesis. We utilized 2-bromoethansulfonate as a positive control for resistance development in our assay system and demonstrated that NFP inhibited in vitro CH_4 production following continuous exposure for 90 d. To our knowledge, NFP is the only compound that specifically inhibits methanogenesis and maintains its potency during long-term treatment.

The replacement of brome hay with DDGS suppressed the amount of CH_4 produced per kilogram of digested DM, but an opposite effect was observed when dietary corn was replaced with DDGS. Both observations can be attributed to the greater methanogenic potential of structural carbohydrates relative to non-sturctural

carbohydrates and possibly the inhibitory effects of dietary fat. Despite changes in CH_4 production, the relative abundance of total ruminal Archaea was not observed to be affected by diet. These results indicate that diet composition may allow for an improved prediction of ruminal CH_4 production compared to the abundance of Archaea.

The replacement of brome hay with corn for 70% of the diet resulted in a decrease in ruminal CH_4 production. Furthermore, the abundance of total Archaea and Methanomicrobiales was suppressed. However, the abundance of Methanobacteriales was increased when corn was substituted for brome hay. Therefore, the decrease in methanogenesis caused by feeding corn rather than brome hay appears to be associated with a decrease in the prevalence of Methanomicrobiales. Lastly, we demonstrated that frequent bolus treatment of BES allowed the treatment to remain effective for 6 d.

Taken together, the work presented in this dissertation furthers our knowledge of how ruminal methanogenesis can be attenuated. We have identified a treatment that remains effective against ruminal methanogens after chronic administration. Several in vivo efficacy trials would be required before a definitive conclusion could be drawn, but the possibility exists for NFP to become a commercially available treatment for reducing ruminal methanogenesis. Furthermore, we have demonstrated that coproducts from corn-ethanol production may be used to reduce CH_4 emissions from ruminant animals consuming a forage-based diet, a feeding strategy that would be most applicable to reducing CH_4 emissions from grazing animals.

Chapter VI

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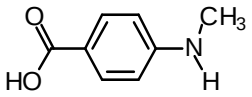
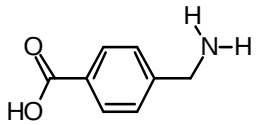
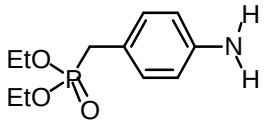
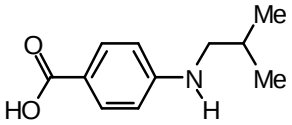
APPENDIX I

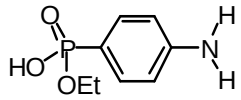
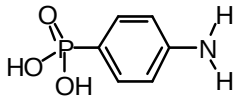
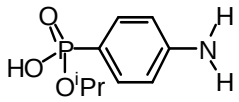
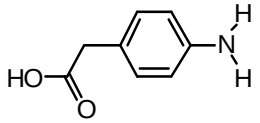
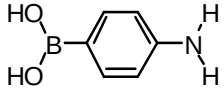
Inhibition of methanogenesis in short-term ruminal cultures by anti-methanogenic compounds

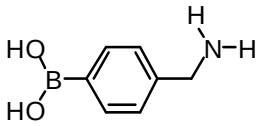
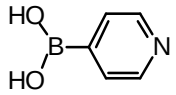
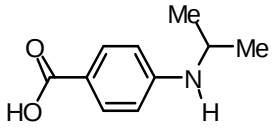
MATERIALS AND METHODS

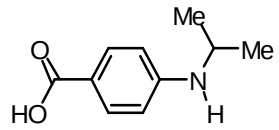
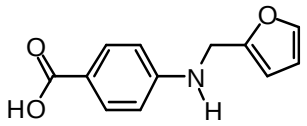
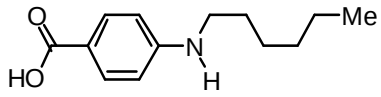
Fresh ruminal fluid was strained through 4 layers of cheesecloth and was used to prepare fermentation media. The fermentation media were composed of 100 mL of McDougall's buffer ((McDougall, 1948); 0.1 M $NaHCO_3$, 0.02 mM Na_2HPO_4, 8 mM KCl, 8 mM NaCl, 0.5 mM $MgSO_4$•$7H_20$, and 1 mM $CaCl_2$•$2H_20$), 100 mL of distilled H_2O, 53 mL of ruminal fluid, 0.5 g of cellobiose, 0.5 g of trypticase, 0.25 g of resazurin, 25 µL of a micro mineral solution (0.5 M $MnCl_2$•$4H_20$, 0.04 M $CoCl_2$•$6H_2O$, and 0.3 M $FeCl_3$•$6H_2O$), and 0.25 g of Na_2S. Anti-methanogenic compounds were dissolved in the indicated solvent and 50 µL of this solution was added to in quadruplicate to 9.4 mL glass vials. While culture media were bubbled with CO_2, oxygen-free gas (H_2/CO_2, 80:20) was projected into each vial with a blunt needle as 4.0 mL of fermentation medium was added to each vial. The vials were then sealed, purged with CO_2, pressurized to 100 kPa above atmospheric pressure, and placed in a shaking incubator (39°C) for 22 h. Following incubation, cultures were cooled to room temperature and the headspace pressure was measured using a micromanometer. Concentration of CH_4 was assayed with a Varian 3700 gas chromatograph (Varian, Inc., Palo Alto, California) fit with a thermal conductivity detector and a silica gel 60/80 grade packed column. Injector, detector, and column temperatures were 120, 120, and 60°C, respectively.

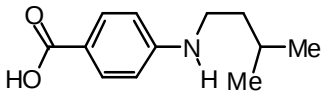
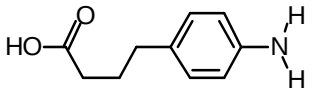
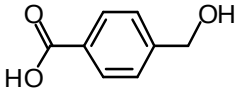
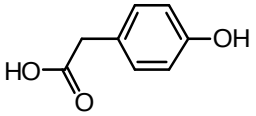
Arithmetic means and standard deviations are reported. Significance of inhibition is indicated by "†" ($P < 0.10$) or "*" ($P < 0.05$).

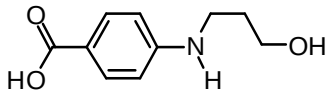
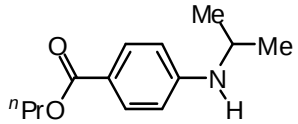
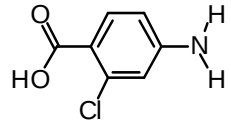
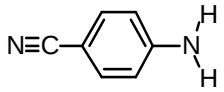
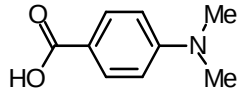
ID	Structure	Assay Date	Concentration (mmol)	Solvent	CH_4 (µmol in treated cultures)	S.D.	CH_4 (µmol in contol cultures)	S.D.	Inhibition (%)	Statistical Difference
I001										
		11/17/03	5.0	DMSO	44.87	0.51	47.83	2.87	**6.2%**	
I005										
		12/18/03	5.0	DMSO	40.57	7.76	42.54	8.58	**4.6%**	
I006										
		12/15/03	5.0	DMSO	61.96	13.30	64.67	9.85	**4.2%**	
I009										
		10/31/03	5.0	DMSO	52.96	3.44	53.02	3.14	**0.1%**	
		12/12/03	5.0	DMSO	30.89	4.44	30.65	5.63	**-0.8%**	

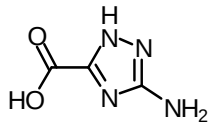
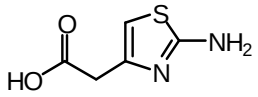
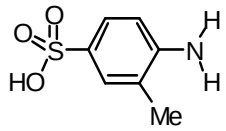
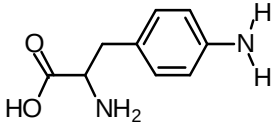
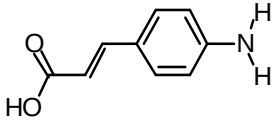
I010										
		12/10/03	5.0	DMSO	52.12	4.40	40.46	5.06	**-28.8%**	*
I011										
		12/18/03	5.0	DMSO	52.37	6.11	42.54	8.58	**-23.1%**	†
I014										
		12/15/03	5.0	DMSO	64.92	8.64	64.67	9.85	**-0.4%**	
I017										
		12/10/03	5.0	DMSO	26.48	1.86	40.46	5.06	**34.6%**	*
		1/22/04	5.0	DMSO	29.67	2.90	44.47	2.43	**33.3%**	*
I018										
		2/7/04	5.0	DMSO	45.45	5.62	36.63	8.16	**-24.1%**	†

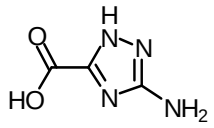
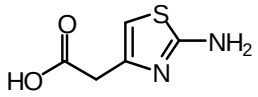
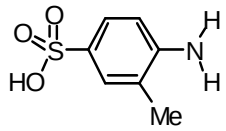
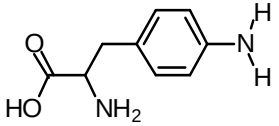
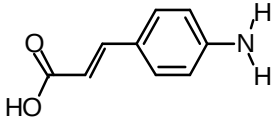
I019										
		9/26/03	0.1	d H_2O	-12.5%		100.0%		**-12.5%**	
		9/26/03	1.0	d H_2O	-14.9%		100.0%		**-14.9%**	
		9/26/03	5.0	d H_2O	6.0%		100.0%		**6.0%**	
		9/26/03	10.0	d H_2O	18.0%		100.0%		**18.0%**	
I020										
		12/18/03	5.0	DMSO	39.91	5.88	42.54	8.58	**6.2%**	
I021										
		10/2/03	0.1	Buffer	81.10	3.46	81.68	6.07	**0.7%**	
		10/2/03	1.0	Buffer	83.94	7.93	81.68	6.07	**-2.8%**	
		10/2/03	10.0	Buffer	58.18	0.94	81.68	6.07	**28.8%**	*
		12/2/03	5.0	DMSO	73.26	5.32	73.60	10.20	**0.5%**	
		2/7/04	5.0	CH_3OH	66.81	4.32	48.56	12.88	**-37.6%**	*
		2/7/04	5.0	DMSO	37.04	4.30	36.63	8.16	**-1.1%**	

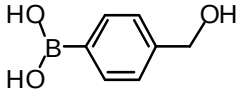
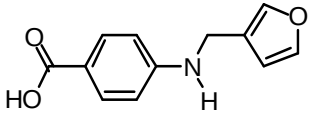
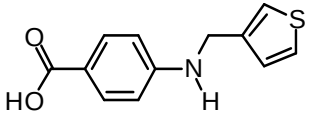
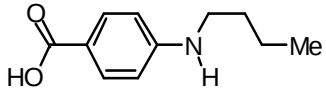
I022										
		12/2/03	5.0	DMSO	88.31	5.81	73.60	10.20	**-20.0%**	*
I023										
		11/11/03	5.0	DMSO	22.57	31.48	70.87	7.99	**68.2%**	*
		12/12/03	5.0	DMSO	1.17	0.66	27.20	3.62	**95.7%**	*
		1/14/04	5.0	DMSO	30.36	1.22	31.50	7.12	**3.6%**	
		2/7/04	5.0	CH_3OH	56.82	6.45	48.56	12.88	**-17.0%**	
		2/7/04	5.0	DMSO	44.77	3.81	36.63	8.16	**-22.2%**	
I024										
		11/17/03	5.0	DMSO	16.29	1.26	47.83	2.87	**65.9%**	*
		3/31/04	5.0	DMSO	16.80	1.88	47.15	4.99	**64.4%**	*
		3/31/04	5.0	DMSO	16.26	1.92	47.15	4.99	**65.5%**	*
		3/31/04	2.5	DMSO	18.32	1.60	47.15	4.99	**61.1%**	*
		3/31/04	1.0	DMSO	28.71	1.87	47.15	4.99	**39.1%**	*
		3/31/04	0.1	DMSO	48.45	4.07	47.15	4.99	**-2.7%**	
		5/19/04	5.0	DMSO	8.61	1.37	33.48	4.94	**74.3%**	*

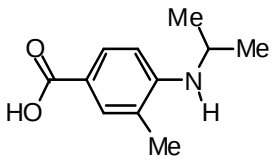
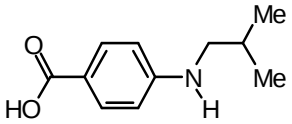
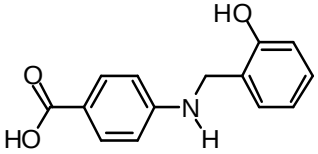
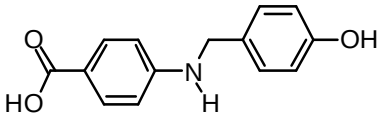
I025										
		10/31/03	5.0	DMSO	29.59	0.68	53.02	3.14	**44.2%**	*
		4/2/04	5.0	DMSO	34.63	0.12	41.19	6.09	**15.9%**	†
		4/2/04	5.0	DMSO	34.79	4.03	41.19	6.09	**15.5%**	†
		4/2/04	2.5	DMSO	35.77	6.00	41.19	6.09	**13.2%**	†
		4/2/04	1.0	DMSO	30.41	3.07	41.19	6.09	**26.2%**	*
		4/2/04	0.1	DMSO	31.39	5.10	41.19	6.09	**23.8%**	*
I031										
		12/15/03	5.0	DMSO	72.48	4.14	64.67	9.85	**-12.1%**	
I032										
		12/15/03	5.0	DMSO	56.48	6.01	64.67	9.85	**12.7%**	
I033										
		12/15/03	5.0	DMSO	50.77	1.39	64.67	9.85	**21.5%**	*

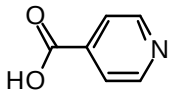
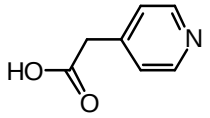
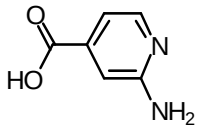
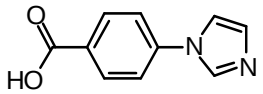
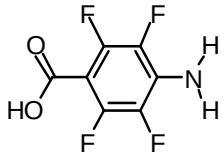
I034										
		2/7/04	5.0	DMSO	33.69	0.54	36.63	8.16	**8.0%**	
I036										
		12/15/03	5.0	DMSO	54.60	13.60	64.67	9.85	**15.6%**	†
I037										
		12/15/03	5.0	DMSO	67.64	10.40	64.67	9.85	**-4.6%**	
I040										
		3/9/04	5.0	DMSO	30.44	1.79	24.77	3.11	**-22.9%**	*
I041										
		11/17/03	5.0	DMSO	41.48	2.46	47.83	47.83	**13.3%**	*
		12/12/03	5.0	DMSO	27.88	2.85	30.65	5.63	**9.0%**	

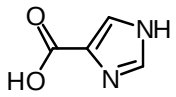
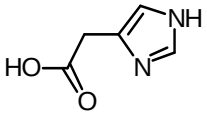
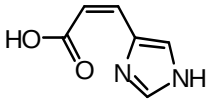
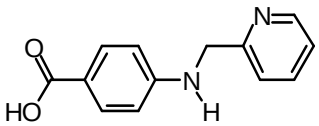
I042										
		12/18/03	5.0	DMSO	44.58	5.96	42.54	8.58	**-4.8%**	
I043										
		12/15/03	5.0	DMSO	58.58	13.30	64.67	9.85	**9.4%**	
I044										
		1/7/04	5.0	DMSO	26.21	3.95	28.52	1.33	**8.1%**	
I045										
		1/7/04	5.0	DMSO	25.06	5.28	27.40	6.27	**8.5%**	
I046										
		2/26/04	5.0	DMSO	21.53	3.29	26.53	3.26	**18.9%**	

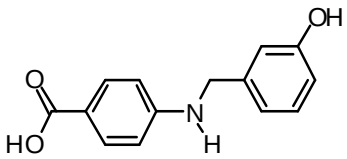
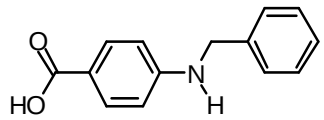
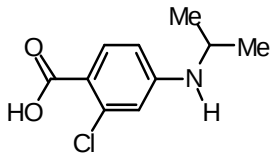
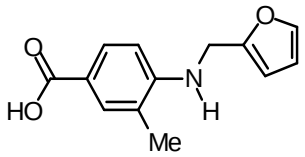
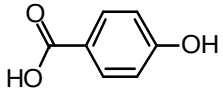
I042										
		12/18/03	5.0	DMSO	44.58	5.96	42.54	8.58	**-4.8%**	
I043										
		12/15/03	5.0	DMSO	58.58	13.30	64.67	9.85	**9.4%**	
I044										
		1/7/04	5.0	DMSO	26.21	3.95	28.52	1.33	**8.1%**	
I045										
		1/7/04	5.0	DMSO	25.06	5.28	27.40	6.27	**8.5%**	
I046										
		2/26/04	5.0	DMSO	21.53	3.29	26.53	3.26	**18.9%**	

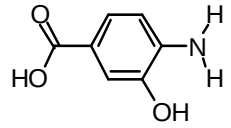
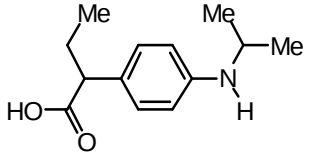
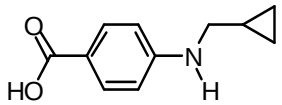
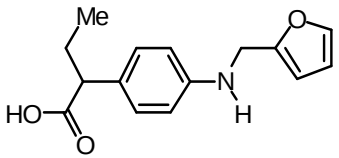
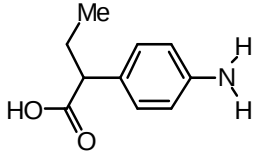
I052										
		10/2/03	0.1	Buffer	80.14	5.31	81.68	6.07	**1.9%**	
		10/2/03	1.0	Buffer	85.84	4.24	81.68	6.07	**-5.1%**	
		10/2/03	10.0	Buffer	58.07	2.39	81.68	6.07	**28.9%**	*
		12/2/03	5.0	DMSO	56.40	3.37	73.60	10.20	**23.4%**	†
I053										
		12/12/03	5.0	DMSO	28.21	4.55	27.20	3.62	**-3.7%**	
I054										
		11/17/03	5.0	DMSO	46.08	4.86	47.83	2.87	**3.7%**	
		11/18/03	5.0	DMSO	49.45	3.55	61.19	8.90	**19.2%**	*
I055										
		11/17/03	5.0	DMSO	45.75	7.14	47.83	2.87	**4.4%**	

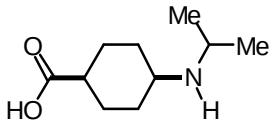
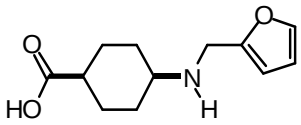
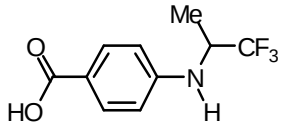
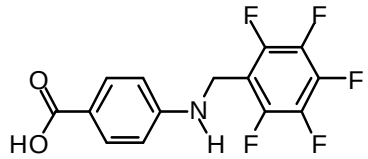
I056										
		12/12/03	5.0	DMSO	29.48	2.32	27.20	3.62	**-8.4%**	
I060 and I009										
		1/7/04	5.0	DMSO	28.45	5.19	28.52	1.33	**0.2%**	
I061										
		2/25/04	5.0	DMSO	21.05	2.23	31.91	0.87	**34.0%**	*
		5/5/04	5.0	DMSO	27.61	4.62	43.76	5.69	**36.9%**	*
		5/5/04	2.5	DMSO	34.47	9.51	43.76	5.69	**21.2%**	*
		5/5/04	1.0	DMSO	36.37	7.23	43.76	5.69	**16.9%**	†
		5/5/04	0.1	DMSO	43.35	3.01	43.76	5.69	**0.9%**	
I062										
		11/18/03	5.0	DMSO	43.51	9.17	61.19	8.90	**28.9%**	*

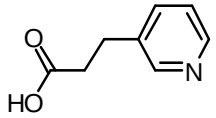
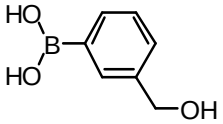
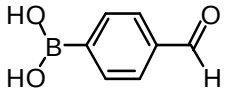
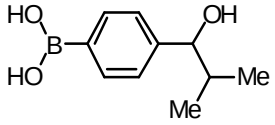
I063										
		1/7/04	5.0	DMSO	26.45	5.77	28.52	1.33	**7.3%**	
I064										
		1/12/04	5.0	DMSO	24.44	3.55	28.14	5.62	**13.1%**	
I065										
		2/26/04	5.0	DMSO	27.86	5.59	26.53	3.26	**-5.0%**	
I066										
		2/26/04	5.0	DMSO	23.92	6.13	26.53	3.26	**9.8%**	
I067										
		1/12/04	5.0	DMSO	26.64	3.16	28.14	5.62	**5.3%**	

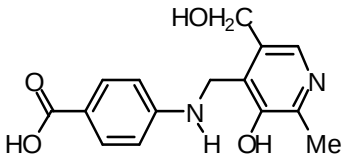
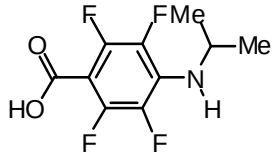
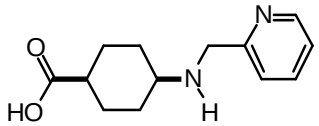
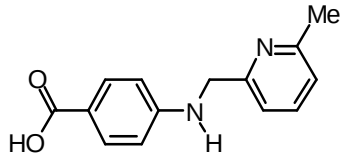
I068										
		1/14/04	5.0	DMSO	46.26	3.16	31.50	7.12	**-46.9%**	*
I069										
		1/14/04	5.0	DMSO	32.64	5.04	31.50	7.12	**-3.6%**	
I070										
		2/26/04	5.0	DMSO	29.13	4.44	26.53	3.26	**-9.8%**	
I071										
		10/1/03	0.1	d H_2O	70.76	5.88	70.07	7.96	**-1.0%**	
		10/1/03	1.0	d H_2O	71.08	10.09	70.07	7.96	**-1.5%**	
		10/1/03	10.0	d H_2O	55.58	9.41	70.07	7.96	**20.7%**	*
		12/2/03	5.0	DMSO	78.13	6.37	73.60	10.20	**-6.2%**	

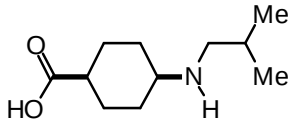
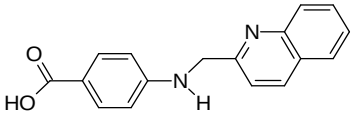
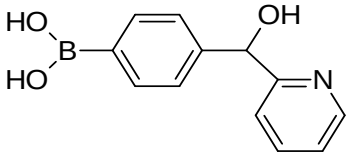
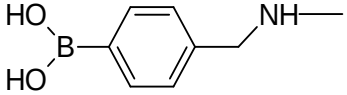
I072										
		10/31/03	5.0	DMSO	43.94	3.53	53.02	3.14	**17.1%**	*
I073										
		11/18/03	5.0	DMSO	48.89	7.25	61.19	8.90	**20.1%**	*
		12/12/03	5.0	DMSO	34.57	1.06	30.65	5.63	**-12.8%**	
I074										
		2/25/04	5.0	DMSO	32.08	1.66	31.91	0.87	**-0.6%**	
I075										
		2/25/04	5.0	DMSO	31.63	1.05	31.91	0.87	**0.9%**	
I076										
		10/31/03	5.0	DMSO	51.26	3.96	53.02	3.14	**3.3%**	

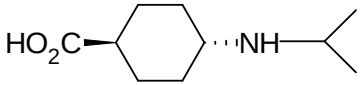
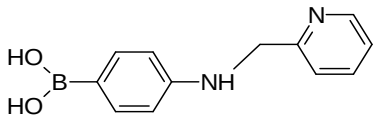
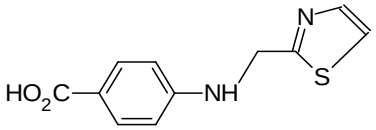
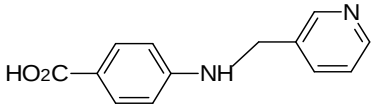
I077										
		1/12/04	5.0	DMSO	29.83	4.64	28.14	5.62	**-6.0%**	
I078										
		2/26/04	5.0	DMSO	25.89	4.97	26.53	3.26	**2.4%**	
I079										
		10/31/03	5.0	DMSO	49.89	4.60	53.02	3.14	**5.9%**	
I080										
		3/24/04	5.0	DMSO	29.42	3.69	35.30	3.97	**16.7%**	*
I082										
		3/24/04	5.0	DMSO	29.23	5.35	33.57	8.31	**12.9%**	

I083										
		3/24/04	5.0	DMSO	31.52	1.72	35.30	3.97	**10.7%**	†
I084										
		12/10/03	5.0	DMSO	24.14	3.05	39.91	0.38	**39.5%**	*
		5/11/04	2.5	DMSO	35.12	2.02	52.01	3.19	**32.5%**	*
		5/11/04	0.75	DMSO	20.00	7.61	52.01	3.19	**61.5%**	*
		5/11/04	0.1	DMSO	56.10	3.40	52.01	3.19	**-7.9%**	
I085										
		12/10/03	5.0	DMSO	40.89	2.37	40.46	5.06	**-1.1%**	
I087										
		11/11/03	5.0	DMSO	27.31	2.93	70.87	7.99	**61.5%**	*
		4/14/04	5.0	DMSO	30.71	1.53	42.80	4.01	**28.2%**	*
		4/14/04	5.0	DMSO	34.33	5.45	42.80	4.01	**19.8%**	*
		4/14/04	2.5	DMSO	43.48	2.38	42.80	4.01	**-1.6%**	
		4/14/04	1.0	DMSO	51.58	2.85	42.80	4.01	**-20.5%**	*
		4/14/04	0.1	DMSO	46.48	11.25	42.80	4.01	**-8.6%**	

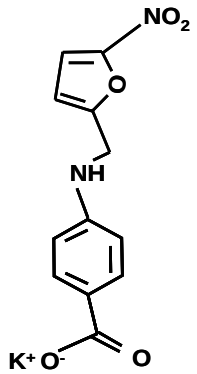
I088										
		3/24/04	5.0	DMSO	33.22	1.29	35.30	3.97	**5.9%**	
I093										
		1/12/04	5.0	DMSO	19.02	0.94	28.14	5.62	**32.4%**	*
		5/5/04	5.0	DMSO	30.31	4.42	43.76	5.69	**30.7%**	*
		5/5/04	2.5	DMSO	32.51	3.53	43.76	5.69	**25.7%**	*
		5/5/04	1.0	DMSO	35.22	5.40	43.76	5.69	**19.5%**	*
		5/5/04	0.1	DMSO	44.32	5.86	43.76	5.69	**-1.3%**	
I094										
		1/14/04	5.0	DMSO	27.78	0.51	31.50	7.12	**11.8%**	
I096										
		11/11/03	5.0	DMSO	39.63	2.27	70.87	7.99	**44.1%**	*
		5/5/04	5.0	DMSO	38.79	5.24	47.13	6.54	**17.7%**	
		5/5/04	2.5	DMSO	40.87	3.90	47.13	6.54	**13.3%**	
		5/5/04	1.0	DMSO	53.86	8.87	47.13	6.54	**-14.3%**	
		5/5/04	0.1	DMSO	59.20	2.70	47.13	6.54	**-25.6%**	*

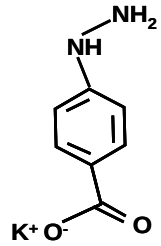
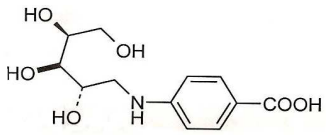
I098										
		3/24/04	5.0	DMSO	33.02	2.68	35.30	3.97	**6.5%**	
I099										
		3/31/04	5.0	DMSO	34.17	6.05	30.88	4.17	**-10.7%**	
I101										
		10/1/03	0.1	d H_2O	77.21	4.15	70.07	7.96	**-10.2%**	
		10/1/03	1.0	d H_2O	63.08	16.27	70.07	7.96	**10.0%**	
		10/1/03	10.0	d H_2O	43.87	8.19	70.07	7.96	**37.4%**	*
I102										
		12/2/03	5.0	DMSO	78.92	5.97	73.60	10.20	**-7.2%**	

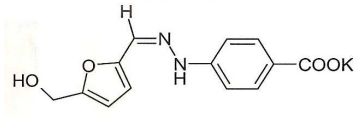
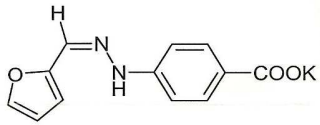
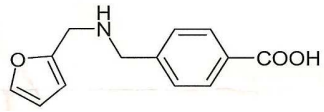
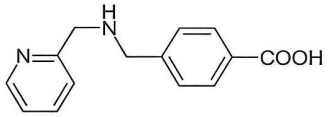
I103										
		12/10/03	5.0	DMSO	37.86	3.82	39.91	0.38	**5.1%**	
I105										
		3/31/04	5.0	DMSO	20.96	2.34	30.88	4.17	**32.1%**	*
I106										
		3/31/04	5.0	DMSO	28.90	2.30	30.88	4.17	**6.4%**	
I107										
		3/9/04	5.0	DMSO	10.42	1.06	24.77	3.11	**57.9%**	*
		5/5/04	5.0	DMSO	38.94	2.96	47.13	6.54	**17.4%**	†
		5/5/04	2.5	DMSO	37.13	4.70	47.13	6.54	**21.2%**	*
		5/5/04	1.0	DMSO	50.62	10.59	47.13	6.54	**-7.4%**	
		5/5/04	0.1	DMSO	46.05	7.51	47.13	6.54	**2.3%**	

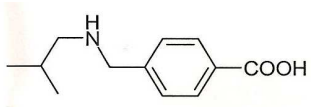
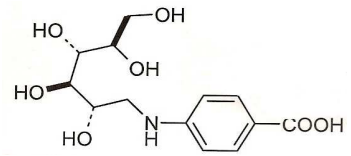
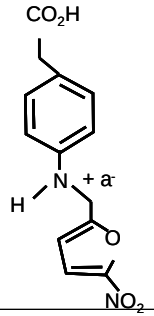
I108										
		3/9/04	5.0	DMSO	17.89	1.71	24.77	3.11	**27.8%**	*
I111										
		3/9/04	5.0	DMSO	15.43	0.50	24.77	3.11	**37.7%**	*
		5/11/04	2.5	DMSO	45.89	8.57	52.01	3.19	**11.8%**	
		5/11/04	1.0	DMSO	48.49	4.00	52.01	3.19	**6.8%**	
		5/11/04	0.1	DMSO	48.56	4.80	52.01	3.19	**6.6%**	
I112										
		3/31/04	5.0	DMSO	27.70	7.23	30.88	4.17	**10.3%**	
I116										
		3/31/04	5.0	DMSO	29.10	5.90	30.88	4.17	**5.7%**	
I123										
		3/9/04	5.0	DMSO	29.75	1.20	29.79	3.82	**0.1%**	
PA-I123										
		5/16/04	5.0	DMSO	27.20	6.21	33.85	7.94	**19.7%**	†

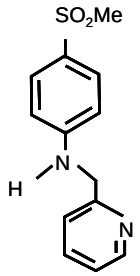
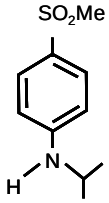
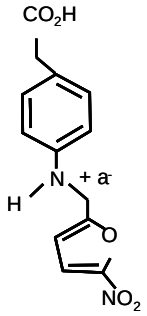
I124										
		3/9/04	5.0	DMSO	17.21	1.32	29.79	3.82	**42.2%**	*
		5/11/04	5.0	DMSO	48.45	4.97	52.01	3.19	**6.8%**	
		5/11/04	2.5	DMSO	47.80	13.89	52.01	3.19	**8.1%**	
		5/11/04	1.0	DMSO	47.15	12.38	52.01	3.19	**9.3%**	
		5/11/04	0.1	DMSO	51.19	5.44	52.01	3.19	**1.6%**	
PA-I124										
		5/16/04	5.0	DMSO	30.16	9.59	33.85	7.94	**10.9%**	
I125										
		3/9/04	5.0	DMSO	27.95	1.43	29.79	3.82	**6.2%**	
PA-I125										
		5/16/04	5.0	DMSO	30.89	1.98	33.85	7.94	**8.7%**	
PA-I126										
		5/16/04	5.0	DMSO	29.85	3.62	33.85	7.94	**11.8%**	
I127										
		3/9/04	5.0	DMSO	30.39	3.93	29.79	3.82	**-2.0%**	
PA-I127										
		5/16/04	5.0	DMSO	22.43	3.41	33.85	7.94	**33.7%**	*
PA-I128										
		5/16/04	5.0	DMSO	27.30	4.91	33.85	7.94	**19.4%**	*
PA-I129										
		5/16/04	5.0	DMSO	15.67	1.06	33.85	7.94	**53.7%**	*
PA-I130										
		5/16/04	5.0	DMSO	16.71	2.77	33.85	7.94	**50.6%**	*
PA-I131										
		5/16/04	5.0	DMSO	52.94	2.65	53.74	2.80	**1.5%**	
PA-I132										
		5/16/04	5.0	DMSO	53.62	2.91	53.74	2.80	**0.2%**	

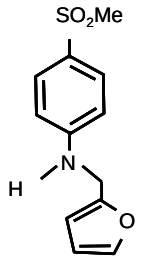
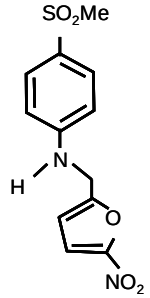
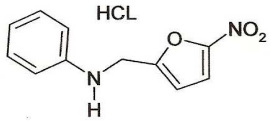
PA-I133										
		5/16/04	5.0	DMSO	0.45	0.52	53.74	2.80	**99.2%**	*
		5/19/04	2.5	DMSO	0.68	0.45	54.57	4.76	**98.8%**	*
		5/19/04	1.0	DMSO	0.84	0.01	54.57	4.76	**98.5%**	*
		5/19/04	0.1	DMSO	44.07	6.87	54.57	4.76	**19.2%**	*
		9/6/05	0.01	DMSO	79.08	5.70	79.06	4.53	**0.0%**	
		9/6/05	0.05	DMSO	68.59	9.21	79.06	4.53	**13.2%**	†
		9/6/05	0.1	DMSO	37.27	4.82	79.06	4.53	**52.9%**	*
		9/6/05	0.5	DMSO	6.25	0.84	79.06	4.53	**92.1%**	*
PA-I134										
		5/16/04	5.0	DMSO	0.00	0.00	53.74	2.80	**100.0%**	*
		5/19/04	1.0	DMSO	6.13	1.80	54.57	4.76	**88.8%**	*
		5/19/04	0.5	DMSO	19.12	2.75	54.57	4.76	**65.0%**	*
		5/19/04	0.1	DMSO	47.47	4.59	54.57	4.76	**13.0%**	*
PA-I135										
		5/16/04	5.0	DMSO	21.90	1.31	53.74	2.80	**59.2%**	*
PA-I136										
		5/16/04	5.0	DMSO	22.88	1.40	53.74	2.80	**57.4%**	*
PA-I137										
		5/16/04	5.0	DMSO	12.59	0.28	53.74	2.80	**76.6%**	*
PA-I138										
		5/16/04	5.0	DMSO	44.45	3.86	53.74	2.80	**17.3%**	*
PA-I139										
		5/19/04	5.0	DMSO	28.79	4.86	33.48	4.94	**14.0%**	†

PA-I140										
		5/19/04	5.0	DMSO	29.46	3.15	33.48	4.94	**12.0%**	
PA-I142										
		5/19/04	5.0	DMSO	0.00	0.00	33.48	4.94	**100.0%**	*
		5/21/04	5.0	DMSO	0.00	0.00	36.48	3.23	**100.0%**	*
		5/21/04	2.5	DMSO	2.75	1.86	36.48	3.23	**92.5%**	*
		5/21/04	1.0	DMSO	5.32	3.31	36.48	3.23	**85.4%**	*
		5/21/04	0.5	DMSO	16.04	0.66	36.48	3.23	**56.0%**	*
		5/21/04	0.1	DMSO	38.98	1.59	36.48	3.23	**-6.8%**	
PA-I143										
		5/21/04	5.0	DMSO	27.59	5.81	29.67	6.63	**7.0%**	
PA-I144										
		5/21/04	5.0	DMSO	28.05	4.80	29.67	6.63	**5.5%**	
PA-I145										
		5/21/04	5.0	DMSO	34.33	5.03	29.67	6.63	**-15.7%**	
PA-I146										
		3/25/04	5.0	DMSO	40.36	7.82	38.31	9.02	**-5.3%**	
PA-I147										
		5/21/04	5.0	DMSO	34.86	2.87	29.67	6.63	**-17.5%**	
PA-I150										
		5/21/04	5.0	DMSO	30.28	4.90	29.67	6.63	**-2.0%**	
PA-I151										
		5/21/04	5.0	DMSO	31.21	6.52	29.67	6.63	**-5.2%**	

PA-I152										
		5/21/04	5.0	DMSO	35.59	4.60	37.33	4.29	**4.7%**	
PA-I153										
		5/21/04	5.0	DMSO	35.25	6.12	37.33	4.29	**5.6%**	
PA-I155										
		3/25/04	5.0	DMSO	30.20	6.48	38.31	9.02	**21.2%**	
		5/21/04	5.0	DMSO	32.26	5.09	37.33	4.29	**13.6%**	
PA-I156										
		3/25/04	5.0	DMSO	34.04	4.27	38.31	9.02	**11.1%**	
		5/21/04	5.0	DMSO	32.91	4.88	37.33	4.29	**11.8%**	
PA-I157										
		3/25/04	5.0	DMSO	37.02	6.82	38.31	9.02	**3.4%**	
		5/21/04	5.0	DMSO	31.44	6.78	37.33	4.29	**15.8%**	†
PA-I158										
		4/21/04	5.0	DMSO	51.90	6.56	52.31	3.50	**0.8%**	
		5/21/04	5.0	DMSO	37.84	1.94	37.33	4.29	**-1.4%**	

PA-I159										
		4/21/04	5.0	DMSO	54.97	3.85	52.20	7.05	**-5.3%**	
		5/21/04	5.0	DMSO	42.92	3.02	37.33	4.29	**-15.0%**	
PA-I160										
		4/21/04	5.0	DMSO	51.23	3.89	52.31	3.50	**2.1%**	
		5/21/04	5.0	DMSO	39.50	5.23	37.33	4.29	**-5.8%**	
62PAL30										
		10/31/05	5.0	DMSO	1.77	0.50	59.50	5.07	**97.0%**	*
		11/16/05	1.0	DMSO	8.16	1.57	78.27	22.83	**89.6%**	*
		11/16/05	0.5	DMSO	21.69	1.81	78.27	22.83	**72.3%**	*
		11/16/05	0.1	DMSO	87.82	5.00	78.27	22.83	**-12.2%**	

62PAL35										
		9/12/05	5.0	DMSO	44.74	0.83	47.09	3.05	**5.0%**	†
62PAL37										
		9/12/05	5.0	DMSO	35.12	1.97	47.09	3.05	**25.4%**	*
62PAL39A										
		9/12/05	5.0	DMSO	1.19	0.48	47.09	3.05	**97.5%**	*
		10/31/05	0.1	DMSO	60.31	16.63	59.50	5.07	**-1.4%**	
		10/31/05	0.5	DMSO	8.25	0.59	59.50	5.07	**86.1%**	*
		10/31/05	1.0	DMSO	0.96	0.75	59.50	5.07	**98.4%**	*

62PAL41A										
		9/12/05	5.0	DMSO	37.08	2.33	47.09	3.05	**21.3%**	*
62PAL43										
		9/12/05	5.0	DMSO	1.70	0.49	47.09	3.05	**96.4%**	*
		10/31/05	0.1	DMSO	16.63	2.56	59.50	5.07	**72.1%**	*
		10/31/05	0.5	DMSO	0.00	0.00	59.50	5.07	**100.0%**	*
		10/31/05	1.0	DMSO	1.93	0.07	59.50	5.07	**96.8%**	*
62PAL68										
		11/16/05	5.0	DMSO	2.55	1.82	78.27	22.83	**96.7%**	*
		11/16/05	1.0	DMSO	0.17	0.35	78.27	22.83	**99.8%**	*
		11/16/05	0.5	DMSO	0.96	0.30	78.27	22.83	**98.8%**	*
		11/16/05	0.1	DMSO	73.60	4.53	78.27	22.83	**6.0%**	
83PAL20										
		8/8/06	5.0	DMSO	75.24	10.69	77.51	12.01	**2.9%**	

83PAL42										
		8/8/06	5.0	DMSO	84.98	10.69	77.51	12.01	**-9.6%**	
83PAL44										
		8/8/06	5.0	DMSO	55.65	3.05	77.51	12.01	**28.2%**	*
83PAL46										
		8/8/06	5.0	DMSO	76.35	8.09	77.51	12.01	**1.5%**	

APPENDIX II

Gas sampling apparatus used for determining methane emission rate