NOVEL STRATEGIES AND COMPOUNDS TO DECREASE RUMINAL METHANOGENESIS IN VITRO

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ABSTRACT

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Novel strategies and compounds to inhibit methane (CH_4) formation in the rumen were evaluated. Inhibition when attempting the inhibition of pyruvate oxidative decarboxylation was small.

Propynoic acid and ethyl 2-butynoate decreased CH_4 production, although they decreased apparently fermented OM (FOM) and resulted in accumulation of H_2, formate, and ethanol. In contrast, ß-hydroxybutyrate, crotonate, and 3-butenoic acid increased FOM.

Aphidicolin and 3-bromopropanesulfonate did not affect CH_4 formation. Lumazine and a novel hexadecatrienoic acid.decreased CH_4 production but decreased FOM. Olive oil did not decrease CH_4 production, but increased propionate production without affecting FOM.

Combinations of lumazine, propynoic acid, and ethyl 2-butynoate with crotonic acid or 3-butenoic acid were hypothesized to improve FOM and decrease accumulation of H_2, formate and ethanol. Crotonic acid and 3-butenoic acid were ineffective in decreasing H_2, formate, and ethanol and improving degradation. Lumazine, propynoic acid, and ethyl 2-butynoate decreased N degradation and increased microbial OM and N production and synthetic efficiencies. Propynoic acid and the highest concentration of ethyl 2-butynoate also decreased OM and NDF degradation.

Differences in sensitivity to inhibitors were found among three ruminal methanogens. *Methanobrevibacter ruminantium* was the most sensitive to 2-bromoethanesulfonate, propynoic acid, and ethyl 2-butynoate, *Methanosarcina mazei* was the least sensitive to those chemicals, and *Methanomicrobium mobile* was intermediate. *M. ruminantium* was the least sensitive to lumazine.

Lumazine caused mild and variable decreases in CH_4 production. It did not impair OM or NDF degradation, and increased microbial N and OM production. It decreased proteolysis, which can increase N retention and decrease N release into the environment. Use of propynoic acid in ruminant diets could be problematic because of potential toxicity. However, it is of interest to understand how it improved microbial synthetic efficiencies. Inclusion of ethyl 2-butynoate in the diet to decrease CH_4 production by about 50% might not affect OM and NDF degradation, decrease proteolysis and increase microbial N flow. Ethyl 2-butynoate toxicity has not been investigated, but it is advantageous that it disappeared after 24 h of incubation. The greatest problem is to rechannel electrons away from H_2, formate, and ethanol into nutritionally useful sinks.

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KEY TO SYMBOLS AND ABREVIATIONS

ADP – adenosine diphosphate

ANOVA – analysis of variance

ATP – adenosine triphosphate

BES – 2-bromoethanesulfonate

BPS – 3-bromopropanesulfonate

C - carbon

CH_4 – methane

CO – carbon monoxide

CO_2 – carbon dioxide

CoA – coenzyme A

CoM or HS-CoM – coenzyme M

CP – crude protein

DE – digestible energy

DM – dry matter

DMI – dry matter intake

DMSO – dimethylsulfoxide

EMNS – efficiency of microbial nitrogen synthesis

EMOMS – efficiency of microbial organic matter synthesis

FOM – apparently fermented organic matter

g = gravity acceleration (9.8 m/s^2)

GC – gas chromatograph

GE – gross energy

GEI – gross energy intake

H – metabolic hydrogen

H_2 – dihydrogen

H_4MPT – tetrahydromethanopterin

HPLC – high precision liquid chromatography

LD_{50} – lethal dose 50

LOI – level of intake

MEI – metabolizable energy intake

MFR – methanofuran

MN – microbial nitrogen

MOM – microbial organic matter

N – nitrogen

N_2 - dinitrogen

NAD^+ - oxidized nicotine adenine dinucleotide

NADH - reduced nicotine adenine dinucleotide

ND – nitrogen degradation

NDF – neutral detergent fiber

NDFD – neutral detergent fiber degradation

NH_4^+ - ammonium ion

Ni - nickel

O_3 – ozone

OD – optical density

OM – organic matter

OMD – organic matter degradation

PMOM – proportion of disappeared organic matter from substrate incorporated into microbial biomass

R^2 – coefficient of determination

RNA – ribonucleotide acid

rRNA – ribosomal ribonucleotide acid

SSU rRNA – small subunit ribosomal ribonucleotide acid

VFA – volatile fatty acids

INTRODUCTION

Ruminants have evolved with a microbial pregastric digestive system that allows the utilization of feed components that are unusable to other animals, such as cellulose, hemicellulose, and non-protein-N (Van Nevel and Demeyer, 1996; Baker, 1997). This is very advantageous, because structural are much more abundant than non-structural polysaccharides (Hobson, 1997). Pregastric microbial digestion allows humans to harvest the photosynthetic potential of grasslands (Russell, 2002), and is the basis for the exploitation of domestic ruminants and camelids for milk, meat, fiber, and draft power (Baker, 1997).

The presence of microbial fermentation between the host animal and plant material, however, constitutes an additional trophic level, and implies inevitable inefficiencies in the transfer of energy and matter (Baker, 1997). Products like CO_2, CH_4, and heat, have no nutritional value for the host animal, and represent a loss of carbon, energy, or both. Methane produced in the foregut and hindgut accounts for between 2 and 15% of the animals gross energy intake, although hindgut fermentation may account for up to 12% of overall CH_4 produced (Czerkawski, 1986; Baker, 1997).

Also, CH_4 production is associated with the balance between glucogenic and non-glucogenic VFA, which can be important for ruminants. Propionate is the main glucose precursor for ruminants, and there is an inverse relationship between propionate molar proportion and the proportion of fermented energy released as CH_4 (Baker, 1997; Wolin et al., 1997).

There is also interest in CH_4 production by domestic ruminants due to the role of CH_4 as a greenhouse gas involved in global warming (Baker, 1997). Methane emissions are responsible for between 18 and 20% of global warming, and ruminal fermentation is a major source of atmospheric CH_4 (Moss, 1993).

For these reasons, considerable research on the inhibition of ruminal methanogenesis has been carried out. Many chemical inhibitors have been investigated, and have proved to be effective in decreasing CH_4 to varying degrees. Problems associated with their use have been decreased animal intake, H_2 accumulation, microbial adaptation, decreased digestibility, toxicity to the host animal, volatility, and inability to improve energetic efficiency (Moss, 1993; Van Nevel and Demeyer, 1996).

Direct inhibition of the pathway of methanogenesis poses the problem of the relocation of the electrons not used in CH_4 formation. Inefficient relocation of these electrons results in H_2 accumulation and decreased re-oxidation of cofactors, which in turn, inhibits fermentation (Van Nevel and Demeyer, 1996). It is proposed in this thesis that alternative strategies, targeted towards inhibiting the production of precursors of CH_4 formation or towards competing with methanogenesis for electrons, may decrease CH_4 production without causing some of those problems.

The present work examines novel strategies and compounds to inhibit ruminal methanogenesis in vitro. Ruminal in vitro techniques allow for a fast, simple, and inexpensive preliminary examination of the effects of chemicals on CH_4 production and fermentation. In Chapter 2, attempts to inhibit pyruvate oxidative decarboxylation, either directly or through the inhibition of thiamin utilization, as a potential means to inhibit CH_4 production by decreasing the supply of precursors, are described. In Chapter 3, the

use of oxaloacetate, butyrate enhancers, and unsaturated compounds as alternative electron sinks to methanogenesis, were studied. In Chapter 4, three compounds that are unrelated in their hypothesized mode of action were examined: aphidicolin, lumazine, and 3-bromopropanesulfonate. In Chapter 5, the effects of a novel hexadecatrienoic fatty acid extracted from a marine algae and of olive oil on CH_4 production and ruminal fermentation were studied.

From Chapters 2 through 5, three compounds were selected based on their ability to decrease CH_4 production: lumazine, propynoic acid, and ethyl 2-butynoate. However, the inhibitors caused the formation of fermentation products without a nutritional value and were estimated to have adverse effects on fermentation. In contrast, two organic acids, crotonic acid and 3-butenoic acid, had minimal effects on CH_4 production but seemed to benefit fermentation. In Chapters 6 and 7, the effects of combinations of lumazine, propynoic acid, and ethyl 2-butynoate, with crotonic acid and 3-butenoic acid, on CH_4 formation, fermentation, substrate degradation, and microbial biomass production were examined. It was hypothesized that the combination of the inhibitors of CH_4 formation with the external electron sinks can relieve the constraints on fermentation caused by the former, and re-channel electrons into butyrate formation.

Adaptation of ruminal microbiota to chemical inhibitors of CH_4 production can also be a problem for their utilization in vivo (Van Nevel and Demeyer, 1996). The presence of methanogens resistant to inhibitors could lead to long term adaptation of the ruminal microbiota (Ungerfeld, 1998). In Chapter 8, the effects of lumazine, propynoic acid, ethyl 2-butynoate, 3-bromopropanesulfonate, and the classical methanogenesis inhibitor 2-bromoethanesulfonate (Nagaraja et al., 1997), on pure cultures of the ruminal

methanogens *Methanobrevibacter ruminantium*, *Methanosarcina mazei*, and *Methanomicrobium mobile* were examined.

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CHAPTER 1

Literature Review

Methane production in the rumen

Ruminal fermentation can be considered an anaerobic oxidation of dietary carbohydrates, proteins, and glycerol, to acetate, CO_2, and NH_4^+, with concomitant production of reduced end products, mainly CH_4, propionate, and butyrate (Van Nevel and Demeyer, 1996). Many of the carbohydrate-fermenting ruminal microorganisms produce H_2, CO_2, and formate as fermentation products; however, none of these organisms produces CH_4. Methanogens use the H_2, CO_2, and formate generated from carbohydrate fermentation to produce CH_4 (Wolin et al., 1997).

More than 90% of the glucose obtained from the degradation of carbohydrates in the rumen is metabolized to pyruvate through glycolysis. As glycolysis releases reducing equivalents, it is essential that pyruvate metabolism provides an electron sink for the reoxidation of the reduced cofactors, so fermentation can continue (Russell and Martin, 1984). Methane formation occurs from pyruvate breakdown products CO_2, formate, and H_2, and provides a route for the disposal of metabolic hydrogen in the absence of oxygen (Stewart et al., 1997). Methanogenesis maintains a low partial pressure of H_2, so that reoxidation of cofactors by hydrogenase is more favorable than by alcohol or lactate dehydrogenase (Van Nevel and Demeyer, 1996). Then, when methanogens are present, CH_4 is the main electron sink. Pyruvate can then be metabolized to acetate, instead of ethanol or lactate, and the ATP yield of the hydrogen producer increases (Figure 1-1; Russell and Wallace, 1997; Wolin et al., 1997):

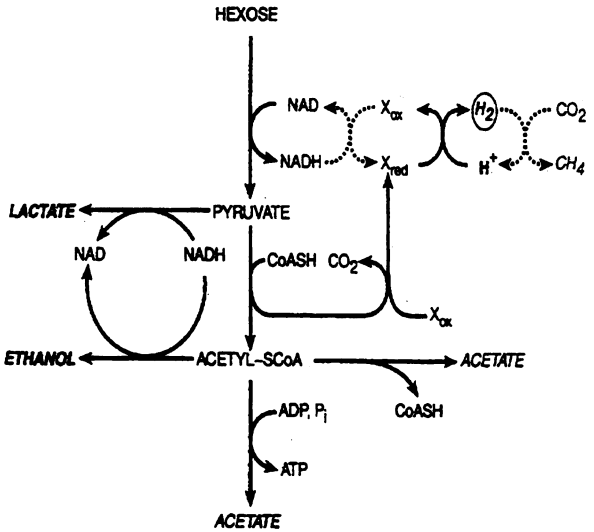

Figure 1-1 Carbohydrate fermentation by an anaerobic ruminal phycomycete in the **absence** and presence of a methanogen (Wolin et al., 1997).

In monoculture, lactate and ethanol are the electron sinks for NADH reoxidation. **In** co-culture with a methanogen, NADH is oxidised to NAD^+ by producing H_2, which is **in turn** used to reduce CO_2 to CH_4 (Wolin et al., 1997).

Most ruminal CH_4 is produced from H_2 and CO_2, although formate can also be **used** as a substrate (Russell and Wallace, 1997). Most formate, however, is converted to **H_2 and** CO_2 prior to methanogenesis (Hungate et al., 1970). Rumen outflow rates are too **high** to allow significant methanogenesis from VFA, as methanogens using these **substrates** grow slowly (Russell and Wallace, 1997).

Although the most important, CH_4 is not the only electron sink in the rumen. Propionate (Russell and Wallace, 1997), butyrate (Miller and Jenesel, 1979), and lactate (Moss, 1993) formations imply the uptake of reducing equivalents. Other electron sinks include sulfite, nitrate, and nitrite (Nagaraja et al., 1997), and fatty acids biohydrogenation and synthesis (Czerkawski, 1986).

Methanogens belong to the domain Archaea, and share with prokaryotes features such as similar size, absence of organelles, and size of ribosomal subunits. Features in common with eukaryotes include cell wall structure, insensitivity to vancomycin, penincillin and kanamycin, absence of formyl-methionine in protein synthesis, and ADP-ribosylation of the peptide synthesis elongation factor EF2 by diphteria toxin. RNA translation and ribosomal shape are distinctive from both prokaryotes and eukaryotes (Moss, 1993; Wolin et al., 1997). All methanogens are fastidious anaerobes, have relatively simple nutritional requirements, and, more importantly, use methanogenesis as the only free energy source for ATP synthesis. Apart from this common physiological features, there is considerable phylogenetic diversity among methanogens, reflected in the macromolecules responsible for the sacculus rigidity, membrane lipid composition, and rRNA sequences. There is variation among methanogens in types and relative amounts of cofactors such as coenzyme F_{420} analogues, vitamin B_{12}-like corrinoids, pterins, and methanofurans (White, 1988; Stewart et al., 1997; Wolin et al., 1997).

For their anabolism, methanogens take advantage of the first part of the CO_2 reductive pathway to de novo synthesize acetate from two molecules of CO_2 and four H_2. Acetyl-CoA is the central metabolite for synthetic reactions. Although the production of acetate from CO_2 and H_2 is thermodynamically favorable in reductive acetogenesis, this

is only at higher partial pressures of H_2 than those found in methanogenic habitats. Furthermore, the formation of acetyl-CoA implies an additional energy cost. However, there is no ATP requirement for acetyl-CoA biosynthesis in methanogens, and the free energy is probably derived from a proton or sodium motive force. Pyruvate can be synthesized from acetyl-CoA, and then converted to glucose by gluconeogenesis. Hexoses are required as building blocks for cell wall components, although glycogen has been found in some methanogens (Blaut, 1994).

Although methanogens as a group are able to use a variety of compounds as N sources, individual species are relatively restricted in their choices. All methanogens can use NH_4^+, many will fix N_2 if deprived from NH_4^+, some can deaminate amino acids, some hydrolize urea, others metabolize methylamines, and some degrade purines or pyrimidines (DeMoll, 1993).

Methanogens are the only archaea that have been found in the rumen. Archaeal small subunit ribosomal RNA (SSU rRNA) comprises between 0.6 and 2.4% of total ruminal SSU rRNA (Lin et al., 1997). Compared to other ecosystems, there have been surprisingly few studies on the isolation and characterization of rumen methanogens. This is partially explained by the difficulties with isolation or culture maintenance (Jarvis et al., 2000). Methanogens that have been isolated from the rumen to date are listed in Table 1-1.

Table 1-1. Ruminal methanogens[1]

	Methanobacterium formicicum	*Methanobrevibacter ruminantium*	*Methanosarcina barkeri*	*Methanosarcina mazei*	*Methanomicrobium mobile*
Morphology	Rods and filaments	Short rods	Irregular cocci	Irregular cocci	Short rods
Dimensions (μm)	0.4-0.8 x 2.0	0.7 x 0.8-1.8	2-3	2-3	0.7 x 1.5-2
Methane from:	H_2/CO_2, formate	H_2/CO_2, formate	H_2/CO_2, acetate, methanol, methylamines	Acetate, methanol, methylamines	H_2/CO_2, formate
Required growth factors	-	Coenzyme M, acetate	-	-	7-mercapto heptanoylthreonine phosphate
Gram reaction	±	+	+	+	-
Motility	-	Variable	-	-	+
G + C in DNA	41-42	31	39	42	49

[1]Adapted from Kuhner et al. (1991) and Stewart et al. (1997).

Species in Table 1-1 are not exclusive to the rumen, and have been isolated from other habitats as well. *Methanobacterium formicicum*, *Methanosarcina barkeri*, and *M. mazei* have been found in ditch muds and sewage plants (Stewart et al., 1997).

Methanogens cell envelopes are different from bacterial. The pseudomurein layer of *Methanobrevibacter* and *Methanobacterium* species does not contain muramic acid, di-aminopimelic acid, or teichoic/teichuronic acids, as does bacterial peptidoglycan (Stewart et al., 1997). The methanogens pseudomurein contains L- instead of D-amino acids, and *N*-acetyl-L-talosaminuronic acid instead of *N*-acetylmuramic acid. Also, the linkage configuration in the glycan strands is β(1,3) rather than β(1,4) as in murein (Sprott and Beveridge, 1993).

Methanogens that do not posses pseudomurein have S-layer proteins, both glycosylated and non-glycosylated. Surrounding the S-layer, *Methanosarcina mazei* has a methanochondroitin layer, composed of a non-sulfated polymer of *N*-acetylgalactosamine, D-glucuronic (or D-galacturonic) acid, some D-glucose, and traces of D-mannose (Sprott and Beveridge, 1993).

The family Methanobacteriaceae, represented in the rumen by *Methanobrevibacter ruminantium,* constituted most of the methanogenic population in a cow rumen (Sharp et al., 1998), and are probably the dominant H_2- and CO_2-using ruminal methanogens (Wolin et al., 1997; Baker et al., 1998). Coccobacilli that morphologically resembled *Methanobrevibacter* spp. and bound antibodies from *Methanobrevibacter smithii* PS and *Methanobrevibacter arboriphilicus* DH1 and DC were isolated from the rumen of a sheep fed silage (Baker et al., 1998). *Methanomicrobium mobile* is the only species of the order Methanomicrobiales so far

found in the rumen. Although it has been isolated on few occasions (Stewart et al., 1997), it is regarded as an important species due to its high numbers (Paynter and Hungate, 1968; Baker et al., 1998), and it was the dominant species in a sheep rumen (Yanagita et al., 2000). The order Methanosarcinales includes in the rumen *Methanosarcina barkeri* and *Methanosarcina mazei*. High numbers of *Methanosarcina* have been found in sheep fed a diet rich in molasses (Rowe et al., 1979), although this has not always been confirmed (Vicini et al., 1987). SSU rRNA from unidentified methanogens of the order Methanococcales has been found in small amounts in the rumen of steers, cows, goats, and sheep (Lin et al., 1997). Other ruminal isolates have not been unequivocally classified (Miller et al., 1986; Stewart et al., 1997; Tokura et al., 1999; Tajima et al., 2001; Takjima et al., 2001).

Some ruminal methanogens have been found to be ecto- (Vogels et al., 1980; Stumm et al., 1982) and endosymbiotically (Finlay et al., 1994) associated with ruminal protozoa. Between 10 and 20% of ruminal methanogens may be ectosymbiotically associated with protozoa (Stumm et al., 1982). Endosymbionts might occupy between 1 and 2% of the protozoon volume (Finlay et al., 1994). Likely, the presence of methanogenic endosymbionts in anaerobic protists is a consequence of the presence of hydrogenosomes. These are organelles engaged in cellular energy metabolism that generate H_2 and acetate from pyruvate or malate (Hackstein and Vogels, 1997). Close physical proximity, but not direct contact, between hydrogenosomes and methanogenic endosymbionts, was found in ruminal protozoa (Wedam et al., 1999). Methanogenic activity of protozoa-enriched fractions of ruminal fluid has been shown to be higher than in strained ruminal fluid (Krumholz et al., 1983). Methane production is generally lower

in defaunated ruminal fluid (Ushida et al., 1997), and in defaunated animals (Whitelaw et al., 1984). Protozoal contribution to total CH_4 of ruminal fluid was estimated to be between 9 and 25% (Newbold et al., 1995), or even 37% in some estimations (Finlay et al., 1994).

Partial sequences of SSU rDNA close to *Methanobrevibacter smithii* were dominant among sequences retrieved from methanogens associated with ruminal ciliates. *M. ruminantium*, however, was absent (Tokura et al., 1999).

Ciliate protozoa affect methanogenesis in a genus-dependent manner. Calves that were re-faunated with different protozoal genus increased their CH_4 production, except for the *Epidinium*-monofaunation, which agrees with the low rate of attachment of methanogens to *Epidinium* sp. (Itabashi, 2001). *Polyplastron multivesiculatum* appears to be only weakly methanogenic, while *Isotricha* sp. could be the predominant CH_4-generating ciliate (Ushida et al., 1997).

Methane production as an energy loss

Despite its profound implication for ruminal fermentation, CH_4 formation is a loss of energy for the host animal, as it is removed by eructation (Trei et al., 1972). Available published data on CH_4 losses by ruminants ranges from 2 to 12% of gross energy intake (GEI). Both extremes occurred in diets with 90% or more concentrates, which causes difficulties with empirical prediction. Other than high grain diets, the majority of practical ruminant diets results in methane losses of 6 ± 0.5% of GEI (Johnson et al., 1993). High grain diets are in general associated with higher amounts of CH_4 as a result

of being more digestible, although the amount of CH_4 produced per unit of OM digested is lower than with roughages.

Among dairy (lactating, pregnant, and dry Holstein cows) and beef cattle (pregnant and dry cows, and steers), sheep, and goats, lactating cows had the highest DM intake (DMI), and the lowest CH_4 production per unit of DMI. Although total CH_4 produced increased with increasing DMI, CH_4 produced per unit of DMI decreased. A quadratic response of CH_4 to DMI was established (Kurihara et al., 1997).

Published values of CH_4 production as a percentage of GEI were used to built multivariate regression models to predict CH_4 energy losses based on level of intake (LOI) and the percentage of digestible energy (DE) in gross energy (GE). Increasing LOI resulted in decreasing CH_4 losses as a percentage of GEI, which was attributed to an increased rate of passage, resulting in decreased ruminal fermentation (Jarosz and Johnson, 1999). As the maintenance subsidy is decreased, the proportion of CH_4 emitted as a consequence of covering maintenance requirements ("non-productive methane losses") decreases as a proportion of total CH_4 losses (Johnson et al., 1993; Kurihara et al., 1997). The relationship of CH_4 losses to DE in GE was much more variable than its relationship to LOI. Digestible energy in GE did little to improve the predictability of CH_4 losses when the diet contained less than 80% roughage. The R^2 values for high forage diets were three times greater than for high grain diets (Hill et al., 1992; Johnson et al., 1993).

Fine grinding and/or pelleting of forage diets can reduce CH_4 losses by 20 to 40%. Also, as the ether extract content increases, CH_4 production decreases (Jarosz and Johnson, 1999).

Energy lost as CH_4 depends on: 1) the fraction of the dietary carbohydrate that is fermented; 2) the VFA profile of the fermentation, as the propionate pathway is an alternative H sink to methanogenesis; and 3) the amount of C and H captured by microbial growth (Johnson et al., 1991; Wolin et al., 1997).

Methane emissions by ruminants and global warming

The solar energy reaching the earth's surface warms it and is radiated back in the infrared region of the spectrum. Approximately 30% of this radiation is absorbed in the troposphere by greenhouse gases: CO_2, CH_4, nitrous oxide, and chlorofluorocarbons. Numerous measurements have shown a recent increase of CH_4 concentrations in the atmosphere. This is well correlated with the increase in human world population, indicating an anthropogenic origin (Moss, 1993).

At present, CH_4 emissions account for about 18% of total global warming. While CO_2 has a greater effect on global warming than CH_4 at the present time, the latter is increasing at a faster rate (1.1 vs. 0.5%/year). Furthermore, as a greenhouse gas, CH_4 is 50 times more potent than CO_2 on a mass basis (Moss, 1993; Young, 2001).

Being a reduced gas in an oxidizing atmosphere, CH_4 must be constantly emitted by some source to be present at a steady state concentration. The chemical reactions that oxidize the atmospheric CH_4 affect the chemical state of the atmosphere through the products of reactions, and the consumption of reactant species. Atmospheric CH_4 exerts an influence on the earth climate both directly and indirectly. The former role involves the absorption of infrared radiation, warming the earth surface, and the near-surface atmosphere, and cooling the stratosphere. Indirectly, CH_4 is ultimately oxidized in the

atmosphere to CO_2. The atmospheric production of CO_2 from CH_4 can be equivalent to about 6% of the direct annual release of CO_2 from anthropogenic sources (Moss, 1993).

Also, the oxidation of CH_4 in the presence of nitrogen oxides produces ozone (O_3), which is a particularly effective greenhouse gas in the upper troposphere. Additionally, the oxidation of CH_4 in the stratosphere produces water vapor, which can cause a temperature increase. Increase in the global average temperature can result in a higher CH_4 production from soil methanogens, accelerating the process (Moss, 1993).

The largest single source of CH_4 (about 21%) appears to be wetlands. About 70% of CH_4 emissions would be anthropogenic (Table 1-2).

Domestic animals appear to rank second among agricultural sources of CH_4, with another 2% possibly contributed by anaerobic decomposition of manure. However, there is uncertainty in these estimates, and enteric emissions from livestock have been ranked as the largest anthropogenic source of CH_4 (McCrabb, 2001).

There are potential sources of CH_4 not listed in Table 1-2. Additional CH_4 in hydrates could be released if melting of the permafrost of the artic tundra begins, or if there is a warming of ocean bottoms. Potential CH_4 release from these sources is very large, and it could become of major importance (Johnson et al., 1991; Moss, 1993).

Table 1-2 Sources of methane emissions[1]

Source	Estimated amount (million ton)	Range (million ton)	Approximate %
Natural wetlands	115	100 – 200	21
Rice paddies	110	60 – 170	20
Domestic animals	75	60 – 95	14
Biomass burning	55	50 – 100	10
Oil and gas drill	45	30 – 50	8
Termites and other wild animals	40	10 – 100	7
Landfills	40	30 - 70	7
Coal mining	35	20 – 45	6
Animal waste	10	5 – 30	2
Oceans and lakes	10	6 – 45	2
Hydrates[2]	5	6 – 100[3]	1

[1]Adapted from Johnson et al. (1991); based on Cicerone and Oremland (1988).

[2]Methane molecules surrounded by rigid cages of water; they are prevalent under the permafrost and beneath the sea in continental margins (Moss, 1993).

[3]Methane in hydrates could be released if global temperature continues to increase (Moss, 1993).

Estimates of CH_4 emissions by different species of mammals are shown in Table 1-3. Due to their size and numbers, cattle are major contributors, accounting for about 74% of total domestic animal emissions. Methane production by wild ruminants is very small when compared with domestic animals (Moss, 1993).

Table 1-3 Estimates of global methane emissions by mammals[1]

Species	Population[2] (million)	Typical methane production (l/d)	Total amount (million ton)	Fraction (%)
Cattle, developed countries	603	210	33.1	41
Cattle, developing countries	688	134	24.1	30
Sheep	1,150	19	6.9	9
Water buffalo	126	192	6.3	8
Wild mammals	237	variable	4.0	5
Goats	460	19	2.3	3
Horses and mules	117	74	1.7	2
Camels	17	223	1.0	1
Pigs	800	5	1.0	1
Humans	4,726	0.2	0.3	0.4
Total			80.7	100.0

[1]Adapted from (Johnson et al., 1991).

[2]Based on 1984 estimates.

Beef cattle are responsible for about two thirds of total cattle emissions in the US. Within beef cattle, beef cows account for about 60% of the emissions (Table 1-4; Johnson, 1993).

Table 1-4 Estimates of methane production by US cattle[1]

Class	Population (million)	Days fed	Energy in methane (% of GE)	CH_4 (L/head /d)	Total CH_4 (million ton/year)	Fraction (%)
Beef cows	33.7	365	6.2	262	2.3	39
Dairy cows	10.7	365	5.8	492	1.4	23
Stockers	38.1	150	6.5	202	0.8	13
Replacements	9.9	365	6.5	220	0.6	10
Feedlot	26.8	140	3.5	153	0.4	6.7
Calves	38.9	210	6.0	53	0.3	5
Bulls	2.2	365	6.0	380	0.2	3.3
Total	160.3				6.0	100

[1]Adapted from Johnson et al. (1991).

Potential CH_4 production from animal manure is large. The amount depends on the amount of manure in lagoons, and on the activity of methane oxidizing microorganisms on the lagoon surface. Limited observations suggests that ruminant manure produce only 2% of its potential level (Johnson et al., 1991). However, anaerobic lagoons are a major target for greenhouse gases mitigation, not only because of CH_4, but also because of nitrous oxide emissions (Johnson et al., 2001). Due to much slower outflow rates than the gastrointestinal tract, acetate is the main precursor for methanogenesis in anaerobic fermentation of animal effluent (Takahashi, 2001).

Biochemistry and energetics of methanogenesis

Most methanogens can grow on CO_2 and H_2 as sole energy sources (Thauer et al., 1993):

$$CO_2 + 4\,H_2 \rightarrow CH_4 + 2\,H_2O$$

The reduction of CO_2 to CH_4 proceeds via coenzyme bound C_1-intermediates. Methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme M (HS-CoM) are the three C_1-unit carriers found in all methanogens analyzed to date. Methanogenesis also involves several electron carriers: coenzyme F_{420}, *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine, ferredoxin, a polyferredoxin with 12 [4Fe-4S] clusters and other ion-sulfur proteins with unknown functions. Methanogens capable of oxidizing methyl groups also contain cytochromes (Thauer et al., 1993).

The following coenzyme-bound C_1-intermediates in methanogenesis from carbon dioxide and hydrogen have been identified (Thauer et al., 1993): N-formyl-MFR (CHO-MFR), N^5-formyl-H_4MPT (CHO-H_4MPT), N^5, N^{10}-methenyl-H_4MPT ($CH{\equiv}H_4MPT^+$), N^5, N^{10}-methylene-H_4MPT ($CH_2{=}H_4MPT$), N^5-methyl-H_4MPT (CH_3-H_4MPT), and methylcoenzyme M (CH_3-S-CoM). The partial reactions and their free energy changes are shown in Table 1-5.

Table 1-5 Partial reactions of methanogenesis and free energy changes

$CO_2 + MFR + H_2 \rightarrow CHO\text{-}MFR + H_2O + H^+$; $\Delta G^{0'} = 16$ kJ/mol

$CHO\text{-}MFR + H_4MPT \rightarrow CHO\text{-}H_4MPT + MFR$; $\Delta G^{0'} = -4.4$ kJ/mol

$CHO\text{-}H_4MPT + H^+ \rightarrow CH{\equiv}H_4MPT^+ + H_2O$; $\Delta G^{0'} = -4.6$ kJ/mol

$CH{\equiv}H_4MPT^+ + H_2 \rightarrow CH_2{=}H_4MPT + H^+$; $\Delta G^{0'} = -5.5$ kJ/mol

$CH_2{=}H_4MPT + H_2 \rightarrow CH_3\text{-}H_4MPT$; $\Delta G^{0'} = -17.2$ kJ/mol

$CH_3\text{-}H_4MPT + HS\text{-}CoM \rightarrow CH_3\text{-}S\text{-}CoM + H_4MPT$; $\Delta G^{0'} = -29.7$ kJ/mol

$CH_3\text{-}S\text{-}CoM + H_2 \rightarrow CH_4 + HS\text{-}CoM$; $\Delta G^{0'} = -85$ kJ/mol

These reactions account for a total free energy change of –130.4 kJ/mol, which differs only by 0.6 kJ/mol from the free energy change calculated from the standard free energies of formation from the elements (Thauer et al., 1993). There is evidence that the last two reactions are coupled with energy conservation by transmembrane proton and sodium ion gradients (Blaut, 1994), and that the first, endergonic reaction, is driven by reversed electron transport (Thauer et al., 1993).

Methanogens contain several hydrogenases for the activation of H_2, which is a substrate in four of the seven partial reactions (Table 1-5). There are two (NiFe) hydrogenases: a coenzyme F_{420}-reducing hydrogenase, and a coenzyme F_{420}-non-reducing hydrogenase, with an unknown electron acceptor. In addition of the two (NiFe) hydrogenases, most methanogens contain a third, very active hydrogenase, H_2-forming methylenetetrahydromethanopterin dehydrogenase, which differs from other

hydrogenases known to date in that it does not contain nickel or ion/sulfur clusters (Thauer et al., 1993).

Control of methane production in the rumen

Strategies to decrease CH_4 production in the rumen include dietary manipulation, chemical additives, microbial additives, and others.

Dietary manipulation

Increasing the proportion of concentrates in the diet usually decreases the proportion of GEI lost as CH_4 (Johnson et al., 1993; Van Nevel and Demeyer, 1996), although no differences were observed in goats fed at maintenance when the percentage of hay in the diet was decreased from 90 to 30% (Kurihara et al., 1997). In lactating dairy cows, however, CH_4 energy losses were decreased from 14.3 to 10.5% of metabolizable energy intake (MEI) when hay in the diet was decreased from 70 to 30% (Kurihara et al., 1997).

Elevation of dietary crude protein from 4 to 9% in goats fed at maintenance resulted in an increase in CH_4 produced per unit of DMI and the ratio of CH_4 energy losses to MEI (Kurihara et al., 1997).

A modified mathematical model of rumen digestion was used to simulate the effect of different nutritional strategies on CH_4 production (Benchaar et al., 2001). It was found that diet changes would allow decreases in CH_4 emission between 10 and 40%. Increasing DM intake and the proportion of concentrate in the diet decreased CH_4 losses as a proportion of GEI by 7 and 40%, respectively. The replacement of fibrous

concentrate with starchy concentrate, and the utilization of less ruminally degradable starch decreased CH_4 losses by 22 and 17%, respectively. The use of more digestible forage resulted in decreases between 15 and 21%. The replacement of legumes for grasses reduced CH_4 losses by 28%, while replacing silage for hay decreased it by 20%.

In general, decreasing methanogenesis is accompanied by a decreased acetate to propionate ratio, in agreement with competing interspecies H transfer reactions between the formation of CH_4 and propionate (Van Nevel and Demeyer, 1996). Means of decreasing the acetate to propionate ratio via the diet include grinding and pelleting of roughages, heat treatment of grain, increasing feeding frequency, increasing intake of mixed diets, and chemical treatments of straws (Van Nevel and Demeyer, 1996).

Supplementation of dry cows with 35 g of $ZnSO_4$ per day decreased CH_4 production per unit of DMI by 62%, and the ratio of CH_4 energy losses to MEI by 61%, although DM digestibility was decreased by 4 percentage units. The reduction in CH_4 production was thought to be related to the repression of rumen fermentation. Although protozoal numbers tended to decrease, methanogens were not affected (Kurihara et al., 1997). It is possible, though, that the most probable number determination used to estimate methanogens numbers only accounted for free methanogens.

Biohydrogenation of fatty acids was proposed as an alternative electron sink to methanogenesis (Czerkawski, 1986). The extent of the inhibition depends on the nature and amount of the lipid fed (Van Nevel and Demeyer, 1996). The extent of the decrease is greater with unsaturated fatty acids (Czerkawski, 1986), and free fatty acids are more potent than triacylglycerols (Van Nevel and Demeyer, 1996). However, the reduction in methanogenesis cannot be explained, at least entirely, by biohydrogentation of fatty

acids, or by negative effects of fatty acids on protozoa (Nagaraja et al., 1997). Studies with pure cultures revealed that ruminal methanogens are very sensitive to long chain, unsaturated fatty acids (Prins et al., 1972; Henderson, 1973). Also, *Ruminococcus albus* and *Ruminococcus flavefaciens*, which have a Gram positive cell wall structure and produce CH_4 precursors, are more inhibited by long chain, unsaturated fatty acids than Gram positive bacteria important in propionate formation (Henderson, 1973; Maczulak et al., 1981). The toxic action of long chain fatty acids is due to adsorption onto the cell wall, which alters nutrients passage (Henderson, 1973).

Methane emissions are also decreased by high amounts of lipids in the diet because of lower ruminal digestion of OM and fiber. Although there can be some compensation by a shift of digestion to the lower tract, the amount of CH_4 formed per mole of substrate fermented in the hindgut is much lower than in the rumen (Van Nevel and Demeyer, 1996).

Supplementation with coconut oil decreased CH_4 production in the chemostat (Machmüller et al., 1998) and in sheep (Machmüller and Kreuzer, 1998), although there was a tendency to decrease fiber digestibility *in vivo*. Canola and cod liver oils decreased CH_4 production in the chemostat without detrimental effect on DM or NDF disappearance. Methanogen numbers were decreased, but cellulolytic and amylolytic bacteria, or endoglucanase activity were not affected, or were increased, by canola and cod liver oils (Dong et al., 1997). The addition of 3.5% soybean oil to dairy cow diets did not change CH_4 production, although the CO_2 to CH_4 ratio increased from 10.4 to 11.3 (Sauer et al., 1998).

Supplementation of sheep with 5% myristic acid ($C_{14:0}$) decreased CH_4 production by about 50% with a 1:1.5 hay:concentrate diet. The extent of the decrease was lower with a 1:0.5 hay:concentrate diet. With this diet, an increase in dietary calcium released the inhibition of CH_4 production, presumably due to the formation of inactive soaps in the rumen (Machmüller et al., 2001)

Lauric acid ($C_{12:0}$) , but not myristic acid ($C_{14:0}$) or stearic acid ($C_{18:0}$) , decreased CH_4 formation in mixed ruminal batch fermentations. Methanogen numbers were decreased by lauric acid and myristic acid, although the latter did not affect methanogenic activity. Possibly, methanogenic activity per cell increased due the excess of H_2 available. Myristic acid in addition to lauric acid enhanced the inhibitory effect of the latter on both methanogenesis and methanogen numbers, showing synergism with respect to CH_4 formation (Soliva et al., 2001).

Chemical additives

Compounds that inhibit ruminal methanogenesis have three different modes of action: 1) inhibition of microorganisms that produce the precursors for CH_4 production (H_2, CO_2, and/or formate); 2) direct inhibition of methanogens; and 3) alternative electron acceptors that compete with methanogenesis.

In the first category are ionophores (Nagaraja et al., 1997) and defaunating (Itabashi, 2001; Takahashi, 2001) agents, which inhibit bacteria with a Gram-positive cell wall structure, and protozoa, respectively. An indirect consequence is a reduction in CH_4 production (McSweeney and McCrabb, 2001), but they are not specific inhibitors of ruminal methanogenesis.

The extent of methanogenesis inhibition caused by ionophores depends on the dose administered, and substrate incubated or ration fed (Van Nevel and Demeyer, 1996). Monensin has been shown to decrease CH_4 production in vitro (Stanier and Davies, 1981; Sauer and Teather, 1987) and in vivo (Sauer et al., 1998). The decrease is mainly mediated by the inhibition that monensin exherts on H_2 -producing bacteria with a Gram-positive cell wall structure, although there might be some direct effects of monensin on methanogens related to an inhibition of Ni uptake. Conversely, gram negatives are protected by their outer membrane. The inhibition of H_2 -producing bacteria depletes the precursors for CH_4 formation. There is a shift in the microbial population towards less sensitive species that produce more propionate (Van Nevel and Demeyer, 1996; Nagaraja et al., 1997). With this approach, H_2 does not accumulate, and propionate increases at the expense of acetate and butyrate (García-López et al., 1996). These changes decrease the energy lost in CH_4, improving the energy retained in VFA (Nagaraja et al., 1997). However, the inhibition of methanogenesis caused by ionophores in vivo is rather modest (between 10 and 30%). Another problem is that partial adaptation of ruminal microbiota for the decrease in methanogenesis has been found after two weeks of feeding monensin. On the contrary, changes in VFA molar proportions were maintained at the end of long term trials with cattle and sheep. This uncoupling of the long term effect of monensin on methanogenesis and the VFA pattern is not in agreement with the stoichiometry of ruminal fermentation (Johnson et al., 1994; Van Nevel and Demeyer, 1996).

Defaunation reduces CH_4 production in vivo between 20 and 50% (Van Nevel and Demeyer, 1996). Although it increases total bacterial numbers, it decreases methanogens (Itabashi, 2001). Defaunation can decrease methanogenesis by acting at

different levels: less fiber digestion, loss of methanogens attached to protozoa, and loss of protozoa themselves, as they are strong producers of H_2 and formate. At present, no satisfactory defaunation method is available to apply on a practical scale (Van Nevel and Demeyer, 1996).

Because of its selective inhibition of bacterial hydrogenases, CO decreased the availability of H_2, and therefore decreased CH_4 production by 89% (Russell and Martin, 1984). Acetate to propionate ratio was decreased. However, the fact that the inhibition was not alleviated by the addition of H_2, suggests that CO could have also affected methanogens directly.

A second strategy is to use chemicals that are directly toxic to methanogens. Halogenated CH_4 analogues such as chloroform, or bromochloromethane, and related compounds such as chloral hydrate or amichloral (a hemiacetyl of chloral and starch), are potent inhibitors of methanogenesis. Chloral hydrate is converted to chloroform in the rumen, and the latter inhibits methanogenesis by blocking the methyl transfer from B_{12} to coenzyme M. This prevents the formation of methylcoenzyme M, which is necessary for the last step of methanogenesis. Likely, carbon tetrachloride (CCl_4) and bromochloromethane inactivate the same enzyme complex (García-López et al., 1996). Chloroform and chloral hydrate can have acute toxic effects on the animal. When bromomethane and amichloral were fed to animals, intake was reduced between 0 and 13%, but feed conversion was improved between 0 and 11% in seven out of eight studies (McSweeney and McCrabb, 2001). Because of intake reductions, weight gains decreased between 0 and 5%, although one study found a 10% improvement. A recent study with cyclodextrin-coated bromochloromethane, a process that makes it less volatile, showed

some reduction in feed intake. However, as weight gain was not affected, conversion efficiency was improved by 4 and 11% in low and medium quality diets, respectively (McCrabb et al., 1997).

Pyromellitic diimide and some of its derivatives were shown to be potent inhibitors of CH_4 production in mixed ruminal cultures in vitro (Linn et al., 1982). Pyromellitic diimide caused a 97% decrease in CH_4 production in mixed batch cultures, with a 30-fold increase in H_2 accumulation (Martin and Macy, 1985). The acetate to propionate ratio was decreased when the substrate was hay or soluble carbohydrates, but not with a mixture of hay and concentrate. Even though diimide was a potent inhibitor of methanogenesis in vitro, it did not decrease the number of methanogens in vivo. As the increase in H_2 accumulation would indicate that methanogenesis was directly inhibited, it is possible that CH_4 production per methanogen cell was decreased without affecting the total number of methanogens. Methane formation was found when diimide was added to the rumen fluid of animals being fed the chemical, suggesting adaptation of the mixed ruminal microbiota (Martin and Macy, 1985). Partial adaptation to inhibitors of methanogenesis has also been shown for trichloroacetamide (Clapperton, 1974) and trichloroethyl adipate (Clapperton, 1977). This compound caused a transient inhibition of methanogenesis in vivo, but weight gain and feed conversion efficiency of lambs were worsened by feeding the chemical (Clapperton, 1977).

2-Trichloromethyl-4-dichloromethylene benzo[1,3] dioxin-6-carboxylic acid was shown to decrease CH_4 production by 91% in continuous culture. Hydrogen production increased progressively. Net production of acetate, propionate and butyrate was increased by 15, 119, and 6%, respectively. The efficiency of microbial protein synthesis

was decreased by the inhibitor. There was no adaptation of microbial activity to the inhibitor (Stanier and Davies, 1981).

Several other compounds containing trichloromethyl groups were screened for their ability to inhibit CH_4 production in ruminal mixed cultures in vitro (Davies et al., 1982). Some 6-substituted derivatives of 2,4-bis(trichloromethyl)-benzo[1,3]dioxin inhibited methanogenesis when present at low concentrations. Basic substitutions had good inhibitory activity, but large lipophilic groups reduced it. The authors found sustained effects in vivo with the carboxylic acid and carboxamide derivatives. 2,4-Bis(trichloromethyl)-benzo[1,3]dioxin-6-carboxylic acid was effective in reducing CH_4 production in sheep when administered intraruminally over a 5-week period (Davies et al., 1982). In cattle, CH_4 production was decreased throughout the 23 days of the study. There was a trend for lower intakes and weight gains of cattle fed 2,4-bis(trichloromethyl)-benzo[1,3]dioxin-6-carboxylic acid in the first half of a 28-week performance trial. In the last 14 weeks, there was an improvement in the weight gain and feed efficiency compared to the control at the highest dose of the chemical. The conversion of dietary energy into energy retained in the animal was improved (Davies et al., 1982).

Although CH_4 analogues and other halogenated compounds can be severe inhibitors of CH_4 production, there are several difficulties for their use in animal production: H_2 accumulates, still representing an energy loss, digestibility and microbial growth can be impaired, feed intake can be depressed, the inhibition caused by some compounds can be transient, and some inhibitors can have toxic effects on the animal (Van Nevel and Demeyer, 1996).

Forages contain phenolic monomers such as p-coumaric acid and ferulic acid. p-Coumaric acid, phenolic acid, and cinammic acid, but not their hydrogenated analogs, decreased all the measured end products, including CH_4, in ruminal fermentation in vitro (Ushida et al., 1989). These compounds also decreased digestibility (Martin, 1988); therefore, they do not appear to be specific inhibitors of CH_4 production. Rather, they likely decrease metanogenesis through a reduction in the availability of precursors.

A variety of organotin compounds has been shown to be toxic to several methanogens, including a non-ruminal strain of *Methanosarcina barkeri*. The mechanism of toxicity is unknown (Boopathy and Daniels, 1991).

Analogues of some unique cofactors involved in methanogenesis have been assessed for their inhibitory activity on various enzymes of methanogenesis. Methyl-S-coenzyme M reductase catalizes the last two-electron reductive step of the overall eight-electron reduction of carbon dioxide to methane (Wackett et al., 1987):

$$CH_3\text{-S-CoM} + 2\,e^- + 2\,H^+ \rightarrow CH_4 + \text{HS-CoM}$$

2-Bromoethanesulfonate is a structural analog of coenzyme M, and inhibits the reductive demethylation of methyl-S-coenzyme M (Müller et al., 1993). It is a very specific inhibitor of methanogenesis, and non-toxic to almost all other microorganisms (Sparling and Daniels, 1987). Unfortunately, the inhibition has been transient in vivo (Nagaraja et al., 1997).

The H donor in the reduction of methyl-S-coenzyme M to methane is N-(7-mercaptoheptanoyl)threonine0-3-phosphate (HS-HTP), which forms a heterodisulfide with coenzyme M after releasing one electron (Sauer, 1991):

$$CH_3\text{-S-CoM} + \text{HS-HTP} \rightarrow CH_4 + \text{CoM-S-S-HTP}$$

The heterodisulfide is subsequently reduced by one pair of electrons and recycled.

HS-HTP is bound to a UDP-disaccharide through a carboxylic-phosphoric anhydride linkage. There is a UDP-N-acetylglucosamine (UDP-GlcNAc) binding site in methyl-S-coenzyme M reductase. It was found that a periodate cleaved derivative of UDP-GlcNAc inhibits the formation of the heterodisulfide in a reaction with purified components (Sauer, 1991). To date, the effects of UDP-GlcNAc derivatives have not been studied in live methanogens.

Although cyanocobalamin is required for the activation of methyl-S-coenzyme M reductase, high concentrations were found to be inhibitory. The inhibition by cyanocobalamin and other corrins appeared to be a direct effect on the ATP-dependent activation of the methylreductase. The reduction of CO_2 to formylmethanofuran was also inhibited (Whitman and Wolfe, 1987), as this endergonic reaction is energetically coupled to the reduction of the CoM-S-S-HTP heterodisulfide.

9, 10-Anthraquinone at 5.0 ppm decreased CH_4 production by 78, 95, and 83% in in vitro ruminal batch cultures with hay, a mixed, and a high-concentrate substrate, respectively. There was an increase in H_2 accumulation. Acetate molar percentage was decreased and propionate increased. Total VFA concentrations were decreased with hay, but not with the mixed or high-concentrate substrates. Results with continuous culture showed no adaptation of the ruminal microbiota to 9, 10-anthraquinone. However, prolonged feeding in vitro unexpectedly lowered propionate molar percentage. It is reasonable to think that anthraquinone uncouples the electron transfer from cytochrome-linked or membrane-bound ATP synthesis, thus preventing the reduction of methyl coenzyme M to CH_4 (García-López et al., 1996).

In the formation of methylcoenzyme M, a corrinoid prostetic group, typically 5-hydroxy-benzimidazolyl-cobamide, transfers a methyl group to coenzyme M. Iodopropane is a corrinoid inhibitor, and it inhibited CH_4 production in pure cultures of *Methanobacterium thermoautotrophicum*, *Methanobacterium formicicum*, and *Methanosarcina barkeri*. 2-Iodopropane coated with α-cyclodextrin at 0.2 or 0.4 mM initial concentration inhibited methanogenesis in in vitro ruminal batch cultures by 48% and 97%, respectively, increasing H_2 accumulation (Mohammed et al., 2001).

p-Aminobenzoate (pABA) is a natural substrate for 4-(β-D-ribofuranosyl) aminobenzene 5'-phosphate synthetase. Three analogs of pABA inhibited CH_4 synthesis in mixed ruminal cultures (DeMontigny et al., 2002).

A consequence of the direct inhibition of ruminal methanogenesis is an increase in H_2 accumulation. This, in turn, can interfere with the interspecies H transfer, inhibiting the reoxidation of cofactors (Nagaraja et al., 1997), and causing the accumulation of unusual fermentation end products, like ethanol (McCrabb et al., 1997; Wolin et al., 1997). These fermentation pathways are associated with a reduced efficiency of microbial growth (McSweeney and McCrabb, 2001). The adaptive changes of the ruminal microbial community to the inhibition of methanogens are relatively unknown, although a shift of the VFA pattern towards propionate is a consistent response. This is a consequence of the disruption of the interspecies H transfer, and the relocation of part of the reducing equivalents spared from methanogenesis into propionate formation (Nagaraja et al., 1997; McSweeney and McCrabb, 2001). However, the effects of methanogenesis inhibition and the resulting elevated H_2 partial

pressures on cellulolytic numbers have not been studied (McSweeney and McCrabb, 2001).

Reducing reactions can withdraw reducing equivalents from methanogenesis. Alternative electron sinks as nitrate or sulfate have higher reducing potentials than CO_2 (Itabashi, 2001). Ruminal reduction of nitrate present in plants decreases CH_4 production in the rumen, although this benefit is counterbalanced by the formation of nitrite, which can become toxic if it accumulates (Takahashi, 2001).

Compounds in the fermentation pathways that leads to propionate, and other organic acids, have been used as alternative electron sinks to methanogenesis. Aspartate, fumarate, and malate, each at 0, 4, 8, and 12 mM initial concentration, did not inhibit CH_4 production in 24 h in vitro batch cultures (Callaway and Martin, 1996). Malate did not decrease CH_4 production in vitro in the absence of added substrates and with cracked corn, but it inhibited methanogenesis by 28% with soluble starch as a substrate (Martin and Streeter, 1995). Dihydrogen did not accumulate in these experiments. Methane production in vitro was decreased by pyruvate, acrylate, fumarate, and α-ketoglutarate by 8, 14, 8, and 13%, respectively (López et al., 1999). The increase observed in propionate production with added fumarate and acrylate stoichiometrically agreed with the decrease in CH_4 production.

In continuous culture, 33 and 44% of added acrylate and fumarate, respectively, were recovered in propionate (Newbold et al., 2001). Methane formation was decreased by 14 and 28%, respectively. Fumaric acid was added to sheep diets at 0, 20, 40, and 80 g/kg DM (Newbold et al., 2001). Intake was stimulated and digestibility was not affected. Methane production was decreased by 3, 4, and 12% at 20, 40, and 80 g of

fumaric acid/kg DM, respectively. The addition of fumaric acid resulted in lower molar proportions of acetate and butyrate, and higher propionate.

Fumarate metabolism of several ruminal species was studied with pure cultures (Asanuma et al., 1999). *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Veillonella parvula*, *Selenomonas lactilytica*, and *Wollinella succinogenes* utilized most of the fumarate added to the medium. There was a corresponding increase in succinate and/or propionate production, and a slight increase in acetate and butyrate. Except for *Selenomonas* spp., utilization was similar with H_2 or formate as electron donors, indicating the presence of formate dehydrogenases. Other ruminal bacteria utilized smaller amounts of fumarate.

The apparent K_m of methanogens for H_2 was lower than for the fumarate-utilizing bacteria (Asanuma et al., 1999). However, methanogens had a higher K_m when formate was the electron donor. Coculture of methanogens with fumarate-utilizers showed that the addition of fumarate decreased methanogenesis, especially when formate was the electron donor. Among the fumarate-utilizers, *W. succinogenes* was the most effective in decreasing methanogenesis, which agrees with the fact that it had the lowest K_m among fumarate-utilizers for both H_2 and formate.

Malate at 10 or 20 mM initial concentration decreased methanogenesis in ruminal batch cultures by 15 and 20%, respectively (Mohammed et al., 2001). There was a decrease in the acetate to propionate ratio. The addition of malate to ruminal cultures where methane production was inhibited by 2-iodopropane coated with α-cyclodextrin decreased CH_4 production further, and also decreased H_2 accumulation (Mohammed et al., 2001).

Failure of an inhibitor of ruminal methanogenesis to improve productivity may result from a number of causes: diversion of metabolic H into products unusable by the host animal, adverse effects of the compound on diet palatability, toxicity to ruminal microorganisms or the host animal, the length of time that an effective concentration of the compound is sustained in the rumen may be too short, or microbial populations may adapt to the compound (Van Nevel and Demeyer, 1996; Nagaraja et al., 1997; Baker, 1999).

Microbial additives

Addition of chemical additives is not the only means to rechannel the substrates for CH_4 production into alternative products. Acetogenic bacteria, which are found in the hindgut of mammals and termites, produce acetate from the reduction of CO_2 with H_2 (Nagaraja et al., 1997):

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$$

Reductive acetogenesis is an important H sink in hindgut fermentation. Reductive acetogens were also the main H utilizers in newborn lambs, but seemed to be outcompeted by methanogens thereafter (Morvan et al., 1994). Reductive acetogenesis has been suggested as a possible alternative electron sink to ruminal methanogenesis (Mackie and Bryant, 1994; García-López et al., 1996). However, methanogenesis predominates over reductive acetogenesis as an electron sink in the rumen. As methanogenesis is thermodynamically more favorable (Kohn and Boston, 2000), ruminal methanogens have lower thresholds for utilizing H_2. Also, reductive acetogens are not obligative hydrogenotrophs and can use other compounds as energy substrates. In the

hindgut, however, acetogenesis can be an important electron sink. The reasons for these ecological differences between compartments are unknown (McSweeney and McCrabb, 2001).

The inhibition of CH_4 production, with its resultant increase in H_2 partial pressure, could make reductive acetogenesis more thermodynamically favorable, and eliminate methanogens competitive advantage due to their lower H_2 thresholds. The addition of the reductive acetogen *Peptostreptococcus productus* greatly decreased H_2 partial pressure when methanogenesis was inhibited by 2-bromoethanesulfonate. The addition of the acetogen also resulted in an increase in acetate production (Nollet et al., 1997).

Other microbial additives have been studied regarding their effects on ruminal methanogenesis. Addition to mixed ruminal cultures of the lactic producers *Leuconostoc mesenteroides subsp. Mesenteroides*, *Leuconostoc lactis*, or *Lactococcus lactis subsp. lactis*, or the yeasts *Trichosporon sericeum*, or *Candida kefyr*, were shown to decrease CH_4 production (Gamo et al., 2001).

Others

Genetic selection to improve feed conversion efficiency could decrease CH_4 emissions (Hegarty, 2001), if the level of production was kept constant.

Circulating antibodies against several ruminal microorganisms, including methanogens, were found in Australian sheep The use of a vaccine against ruminal methanogens is currently being investigated as a possible strategy for reducing CH_4 emissions. (McSweeney and McCrabb, 2001).

A brush that mechanically stimulates ruminal motility decreased CH_4 production between 63 and 71%. Apparently, the physical stimulation increased the rate of passage, which decreased CH_4 production. Methane production was not affected by the size of the stimulating brush (Matsuyama et al., 2001).

Conclusions

Although CH_4 production in the rumen has been inhibited by several additives, there have been shortcomings such as transient effects, toxicity, decreased intake and/or digestion. Ruminal fermentation is an interactive network of chemical reactions, and the inhibition of methanogenesis should not be considered as an isolated, specific intervention. Rather, it will have consequences on general microbial activities in the rumen and animal metabolism. An ideal inhibitor must be extremely specific with a persistent action, harmless to the animal and the environment, and without residues in edible products (Van Nevel and Demeyer, 1996). Ultimately, the management of H dynamics in the rumen is the most important factor to be considered when developing strategies to control ruminant CH_4 emissions (Joblin, 1999).

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CHAPTER 2

Attempts to decrease ruminal methanogenesis through the inhibition of pyruvate oxidative decarboxylation

Abstract

The inhibition of pyruvate oxidative decarboxylation was studied as a means of decreasing ruminal methanogenesis in vitro. In Experiment 1, the addition of thiamin (10 mM), amprolium (10 mM), adenine (10 mM), or adenosine (10 mM) decreased methanogenesis by 22, 42, 47, and 76%, respectively. However, microbial growth was almost non-existent, and the ratio of CO_2 to CH_4 in the control was unusually high. Organic matter fermentation was low in all treatments. Most likely, using an isolated cell pellet instead of ruminal fluid, biased the conditions to make them not typical of ruminal fermentation. In Experiment 2, the addition of adenosine (10 mM) and adenine (10 mM), with and without ribose (10 mM), to mixed batch cultures including ruminal fluid did not decrease methanogenesis. In Experiment 3, the addition of oxythiamin (5 mM) decreased methanogenesis by 23%. In Experiment 4, three pyruvate derivatives (2 mM) did not inhibit methanogenesis, although hydroxypyruvate improved OM fermentation by 11%. The strategies employed did not seem to inhibit pyruvate oxidative decarboxylation, and when methanogenesis decreased in Experiment 1, this seemed to be due to the technique used rather than to the treatments imposed.

Introduction

Methane production by ruminants is a carbon and energy loss, and contributes to global warming. The inhibition of CH_4 production in the rumen would have significant economic and environmental benefits (Moss, 1993).

Pyruvate oxidative decarboxylation is the first step in the conversion of pyruvate to acetate and butyrate in the rumen (Russell and Wallace, 1997). This reaction produces acetyl-CoA, CO_2, and reducing equivalents, when catalyzed by pyruvate oxidoreductases (Uyeda and Rabinowitz, 1971), and in acetyl-CoA and formate, when catalyzed by formate lyases (Gottschalk and Andressen, 1979). Reducing equivalents generated by pyruvate oxidative decarboxylation and by glycolysis can be used by methanogens to reduce CO_2 and formate to CH_4 (Moss, 1993).

Thiamin pyrophosphate is a cofactor of pyruvate oxidoreductases (Williams et al., 1990). Thiamin is synthesized in the rumen (McDonald et al., 1995) and is required by or stimulative for some ruminal microorganisms (Wolin et al., 1997). The inhibition of thiamin utilization by ruminal microorganisms could block pyruvate oxidative decarboxylation, diverting pyruvate to propionate formation, decreasing the availability of CO_2 and reducing equivalents for methanogenesis.

Thiamin structural analogs can inhibit bacterial growth (Koser, 1968) and impair thiamin uptake in protozoa (James, 1980; Shigeoka et al., 1987) and animal hepatocytes (Lumeng et al., 1979), erythrocytes, and ghosts (Casirola et al., 1990). Adenine and adenosine impaired thiamin synthesis in *Escherichia coli* (Iwashima et al., 1968) by lowering the hydroxymethylpyrimidine moiety synthesis (Kawasaki et al., 1969).

The objective of this series of experiments was to decrease CH_4 production by mixed ruminal cultures by blocking pyruvate oxidative decarboxylation. In a first experiment, it was hypothesized that the combination of thiamin structural analog amprolium, with adenine or adenosine, would decrease methanogenesis by simultaneously blocking thiamin intracellular synthesis and its extracellular uptake. In the second experiment, adenine and adenosine were hypothesized to decrease CH_4 production by inhibiting thiamin synthesis. In the third experiment, thiamin structural analogs amprolium and oxythiamin were hypothesized to decrease CH_4 production by impairing thiamin utilization. In the fourth experiment, it was hypothesized that the direct inhibition of pyruvate oxidoreductases through the use of pyruvate derivatives (Flournoy and Frey, 1989; Williams et al., 1990) would decrease CH_4 production.

Material and Methods

Experiment 1

Arrangement of treatments. Amprolium, a structural analog of thiamin, was used in conjunction with adenine or adenosine to attempt the simultaneous inhibition of thiamin intracellular synthesis and external thiamin utilization. A basal, fermentation medium without thiamin was used. The 2 x 2 x 3 factorial arrangement of treatments was: 1) thiamin 0 or 10 mM (thiamin effect); 2) amprolium plus (10 mM) or minus (structural analog effect); 3) adenine (10 mM) , adenosine (10 mM) or control (intracellular synthesis inhibition effect).

Ruminal fluid collection and preparation. Ruminal fluid was withdrawn from two mature Holstein cows fed alfalfa hay, mixed, and strained through two layers of

cheese cloth. It was blended for 15 s under O_2 free-CO_2 and then filtered through one layer of cheesecloth. A cell washing procedure was then used to eliminate thiamin present in the liquid phase of the ruminal fluid. Ruminal fluid was centrifuged at 300 x *g* and 4 °C for 10 min in capped tubes under CO_2. The pellet was discarded and the supernatant centrifuged at 20,000 x *g* and 4 °C for 20 min. The supernatant was discarded and an equal volume of buffer (Bryant and Burkey, 1953), previously autoclaved and anaerobically prepared, was used to resuspend the cell pellet. The last step was repeated, and 1 mL of the cell suspension was anaerobically delivered into 25-mL Hungate tubes.

Media preparation. Hungate tubes contained 15 mL of an autoclaved, ruminal fluid-free, thiamin-free medium (Table 2-1), sealed with a rubber stopper under an O_2 free-CO_2 atmosphere. Forty-one milligrams of adenine [Sigma A 8626] and 21 mg of adenosine [Sigma A 9251] were added as solids to the corresponding tubes before delivering the medium, so as to achieve final concentrations of 10 mM. Riboflavin and thiamin were not autoclaved with the other vitamins in order to avoid their destruction. A 0.75 ppm riboflavin solution was prepared, filter-sterilized, and used to deliver thiamin hydrochloride [Sigma T 4625] and amprolium [Sigma A 0542] to the corresponding tubes by anaerobically injecting 0.5 mL into each tube, so as to achieve final concentrations of 10 mM.. Controls received 0.5 mL of the riboflavin solution without thiamin hydrochloride or amprolium.

Table 2-1. Ruminal fluid-free medium used in Experiment 1

Ingredient	Amount (g/L or mL/L)
Cellobiose	1.0
Soluble starch	1.0
Xylose	0.5
Arabinose	0.5
Vitamin-free amino acids[1]	5.0
NaCl	2.0
KH_2PO_4	3.0
K_2HPO_4	3.0
$(NH_4)_2SO_4$	1.0
$CaCl_2$	0.2
$MgSO_4$	0.4
Resazurin solution	2.0
Cysteine sulfur solution[2]	80
Trace mineral solution[3]	0.2
Valeric acid	0.03
Isovaleric acid	0.03
Isobutyric acid	0.03
Sodium acetate	1.968
Vitamin solution[4]	0.2

[1]Vitamin-free Casaminoacids, Difco Laboratories.

[2]L-Cysteine HCl, 2.5 g and $Na_2S \bullet 9H_2O$, 2.5 g were added to deionized water to give a final volume of 200 mL, and pH was adjusted to 6.5 with 3 N NaOH. Cysteine sulfur solution and $NaHCO_3$ were added after boiling the medium twice as described by Butine and Leedle (1989).

[3]H_3BO_3, 620 ppm; $ZnCl_2$, 682 ppm; $MnCl_2 \bullet 4H_2O$, 930 ppm; $CoCl_2 \bullet 6H_2O$, 950 ppm; $Na_2MoO_4 \bullet 2H_2O$, 360 ppm; Na_2SiO_3, 122 ppm; Na_2SeO_3, 173 ppm; $NiCl_2 \bullet 6H_2O$, 130 ppm; $Na_2WO_4 \bullet 2H_2O$, 3 ppm; $Al_2(SO_4)_3$, 0.03 ppm.

[4]Pyridoxamine, 1500 ppm; folic acid, 500 ppm; p-aminobenzoic acid, 300 ppm; biotin, 100 ppm; cobalamin, 100 ppm; hemin, 1000 ppm.

Incubation. Tubes were incubated in a shaking waterbath for 24 h, and optical density measured at 600 nm every 6 h to assess microbial growth. At the beginning of the experiment, three samples of medium with the added cell suspension were frozen for subsequent determination of VFA initial concentrations. At the end of the incubation, tubes were allowed to cool to room temperature, and total gas production was measured as described by (Callaway and Martin, 1996). Fermentation was stopped by adding 1 mL of 12 N H_2SO_4.

Analytical procedures. Methane and CO_2 were analyzed as described by Callaway and Martin (1996), using a Gow Mac series 750 flame ionization detector gas chromatograph (Gow Mac Instruments Co., Bridgewater, NJ) equipped with a 4' x 1/4" DC 200 column, s.s. (150 °C, carrier gas was N_2 at 820 Kpa). A RGD2 Reduction Gas Detector (Trace Analytical, Menlo Park, CA) equipped with the same type of column was used for H_2 analysis. Gas production was expressed as μmoles at 25 °C and 1 atm. A 5-mL aliquot of the fermentation medium was centrifuged (26,000 x *g*, 4 °C, 30 min). Volatile fatty acids, lactate, formate, and ethanol were quantified by differential refractometry with a Waters HPLC (Waters Associates Inc., Milford, MA) equipped with a BioRad HPX 87H column (BioRad Laboratories, Hercules, CA). Solvent was 0.005 M H_2SO_4 at 0.6 mL/min. Column temperature was 65 °C. Sample injection volume was 15 μL. Ammonia was analyzed as described by (Chaney and Marbach, 1962).

Calculations. Apparently fermented OM (FOM) was estimated from the VFA stoichiometry (Demeyer and Van Nevel, 1979), but using isobutyrate instead of caproate:

fermented OM (mg of hexose) = (Acetate/2 + Propionate/2 + Isobutyrate + Butyrate + Valerate + Isovalerate) x 162, with all VFA expressed in mmoles produced.

Statistical analysis. Six replicates per treatment were used. Data from five tubes (all from different treatments) were discarded because their dark color indicated lack of reducing conditions. The experimental model was: response = overall mean + thiamin + amprolium + N base + thiamin x amprolium + thiamin x N base + amprolium x N base + thiamin x amprolium x N base + residual. Data were tested for homogeneity of variances using the Modified Levene's test (Neter et al., 1996) and analyzed as a three-way ANOVA, when homogeneity of variances was not rejected ($P > 0.05$). Probabilities of effects were calculated using Type III sums of squares for an unbalanced design. If homogeneity of variances was rejected, a Kruskal-Wallis test (Neter et al., 1996) was conducted. When significant ($P < 0.05$) effects were found by the ANOVA or the Kruskal-Wallis tests, and in the absence of significant ($P < 0.05$) interactions, factor level means were compared through the Spjovoll/Stoline test for unequal N (Chew, 1976). If the interactions were significant, treatment means of one factor within another were compared through the Spjovoll/Stoline test for unequal N. When the intracellular thiamin synthesis inhibition effect was significant ($P < 0.05$), preplanned contrasts tested were: 1) control vs average of adenine and adenosine, and 2) adenine vs adenosine. The responses of optical density to time were modeled as fourth order polynomials (Neter et al., 1996).

Experiment 2

The effects of adenine and adenosine on CH_4 production and fermentation were evaluated in batch cultures in vitro, with a ruminal fluid/buffer medium. The effects of adenosine's ribose moiety alone or with adenine were also tested. The experimental treatments were: 1) Control; 2) Adenine; 3) Ribose; 4) Adenine + ribose; 5) Adenosine. Ruminal fluid was collected, strained and blended as in Experiment 1, and one part of ruminal fluid mixed with four parts of buffer (Goering and Van Soest, 1975). At the beginning of each experiment, three samples of ruminal fluid and buffer mixture were frozen for subsequent determination of VFA initial concentrations. Fifty milliliters of ruminal fluid and buffer mixture were anaerobically delivered into 125-mL Wheaton bottles. Each bottle had 600 mg of ground (0.2 mm screen) alfalfa hay (11.4% CP in the DM) as substrate, and adenine [Sigma A 8626], ribose [Sigma R 7500], adenine and ribose, or adenosine [Sigma A 9251] added so as to achieve 10 mM final concentrations of each of the compounds. The bottles were sealed under an O_2 free-CO_2 atmosphere and incubated in a shaking water bath at 39 °C for 24 h. Fermentation was terminated by injecting 3 mL of 12 N H_2SO_4 into each bottle. Total gas production and composition, VFA and NH_4^+ analysis, and the calculation of apparently fermented OM, were done as in Experiment 1.

Four replicates per treatment were used. The experimental model was: response = overall mean + treatment + residual. Data were tested for homogeneity of variances using the Modified Levene's test (Neter et al., 1996) and analyzed as a one-way ANOVA, when homogeneity of variances was not rejected ($P > 0.05$). If homogeneity of variances was rejected, a Kruskal-Wallis test (Neter et al., 1996) was conducted. Planned contrasts

of interest were 1) control vs adenine; 2) control vs adenosine; 3) adenine vs adenosine; 4) ribose vs adenosine; 5) adenine + ribose vs adenosine. A Bonferroni adjustment (Neter et al., 1996) for five non-orthogonal comparisons was used, and significance declared at $P < 0.01$ (for an experimentwise type I error probability of 0.05). All other comparisons were done by the Scheffé test.

Experiment 3

The effects of thiamin structural analogs amprolium and oxythiamin on CH_4 production and fermentation were evaluated in batch cultures in vitro, with a ruminal fluid and buffer medium. The experimental treatments were 1) Control; 2) Oxythiamin 5 mM; 3) Oxythiamin 10 mM; 4) Amprolium 5 mM; 5) Amprolium 10 mM. Ruminal fluid was collected and prepared as in Experiment 2. Delivery of the ruminal fluid/buffer mixture and incubation procedures were the same as in Experiment 2. Each bottle had 200 mg of ground (0.2 mm screen) alfalfa hay (11.4% CP in the DM) as a substrate. One milliliter of 0.255 or 0.510 M solutions of amprolium or oxythiamin were added to the corresponding Wheaton bottles so as to achieve final concentrations of 5 or 10 mM. Controls received 1 mL of deionized water. Fermentation was terminated by injecting 1 mL of a 10% phenol solution, instead of H_2SO_4, as in Experiments 1 and 2, so that final pH could be measured (Digital Benchtop pH Meter, Cole-Parmer Instrument Company, Vernon Hills, IL). Total gas production and composition, VFA and NH_4^+ analysis, and the calculation of apparently fermented OM, were done as in Experiment 1.

The number of replicates, the model used and the statistical analyzes were the same as in Experiment 2. Orthogonal polynomial contrasts for linear and quadratic responses to oxythiamin and amprolium were evaluated (Neter et al., 1996).

Experiment 4

The effects of pyruvate derivatives on CH_4 production and fermentation were evaluated in batch cultures in vitro, with a ruminal fluid and buffer medium. The experimental treatments were: 1) Control; 2) Pyruvate (positive control); 3) Bromopyruvate; 4) Fluoropyruvate; 5) Hydroxypyruvate. Ruminal fluid was collected and prepared as in Experiment 2. Delivery of the ruminal fluid/buffer mixture and incubation procedures were the same as in Experiment 2. Each bottle had 300 mg of ground (0.2 mm screen) alfalfa hay (11.4% CP in the DM) as a substrate. One milliliter of 0.102 M solutions of Na-pyruvate [Sigma P 2256], bromopyruvate [Sigma B 9630], Na-fluoropyruvate [Sigma F 4004] and hydroxypyruvate [Sigma H 9270] were added to Wheaton bottles so as to achieve final concentrations of 2 mM. Controls received 1 mL of deionized water. Fermentation was terminated by injecting 1 mL of a 10% phenol solution. Total gas production was measured, and gas composition analyzed as in Experiment 1. Analysis of VFA and NH_4^+, and the calculation of fermented OM, were done as described above. The additives final concentrations were determined by HPLC along with the VFA.

The number of replicates, the model used, and the statistical analyzes were the same as in Experiment 2. Planned contrasts of interest were 1) control vs Na-pyruvate; 2) control vs average of pyruvate derivatives; 3) Na-pyruvate vs average pyruvate

derivatives; 4) bromopyruvate vs Na-fluoropyruvate; 5) bromopyruvate vs hydroxypyruvate; 6) Na-fluoropyruvate vs hydroxypyruvate. A Bonferroni adjustment (Neter et al., 1996) for six non-orthogonal comparisons was used, so significance was declared at $P < 0.0083$ (for an experimentwise type I error probability of 0.05). Other comparisons of interest were done using the Scheffe test (Neter et al., 1996).

Results and Discussion

Experiment 1

Thiamin or amprolium addition did not influence bacterial growth, as measured through optical density (data not shown) or fermented OM (Table 2-2). However, fermented OM (Table 2-2), and optical density at 6, 12, 18 and 24 h (Figure 2-1), were strongly increased by adenosine, and inhibited by adenine.

Total OM fermentation (OM in medium plus additives) was very low in all treatments, ranging from 5.4 to 21.1% (Table 2-2). There was no increase in optical density in control. When the estimated fermented OM was related to the carbohydrates initially present in the medium only (i.e., ignoring the amino acids and additives), fermented OM was higher, ranging between 30.6 and 81.1% (data not shown). Adenosine addition stimulated ($P < 0.01$) OM fermentation (16.6% vs 10.6% of total OM in control and added adenosine, respectively). Adenine, in contrast, inhibited ($P = 0.02$) OM fermentation (8.4% vs 10.6% of total OM with and without adenine, respectively). It has been shown that adenosine, but not adenine, could support growth of *Prevotella* (*Bacteroides*) *ruminicola* and *Selenomonas ruminantium* as the only energy source

Table 2-2. Effects of thiamin, amprolium, adenine and adenosine on end products of isolated ruminal bacteria fermentation (Exp. 1)

Thiamin, mM	Amprolium, mM	Adenine, mM	Adenosine, mM	n	FOM[1], %	CH_4, µmol	CO_2, µmol	Acetate	Propionate	Butyrate	NH_4^+, mg/dL
								mol/ 100 mol			
0	0	0	0	5	13.8	11.0	808	49.0	38.1	9.7	20.1
0	0	10	0	5	11.4	5.5	746	50.6	40.1	4.1	19.2
0	0	0	10	6	21.1	4.5	783	41.4	54.6	1.1	29.5
0	10	0	0	6	10.2	7.6	238	48.3	31.3	10.4	19.1
0	10	10	0	5	7.8	4.6	1062	48.8	37.5	5.7	17.9
0	10	0	10	6	16.2	2.5	515	41.2	53.5	1.9	29.5
10	0	0	0	6	10.5	10.2	507	53.5	28.6	10.7	18.9
10	0	10	0	6	7.3	5.6	683	56.8	34.0	1.7	16.9
10	0	0	10	6	16.4	3.3	456	39.1	53.3	1.9	27.0
10	10	0	0	6	8.0	5.1	484	47.3	33.1	9.6	16.5
10	10	10	0	4	5.4	2.3	574	47.9	38.9	3.5	15.9
10	10	0	10	6	12.8	1.2	919	40.0	54.0	2.4	26.3
Effects of:						Probabilities[4]					
Thiamin					0.66	0.04	0.59	0.30	< 0.01	0.28	< 0.01
Amprolium					0.99	< 0.01	0.84	< 0.01	0.94	0.11	0.01
Intracellular synthesis inhibitor					< 0.01	< 0.01	0.43	< 0.01	< 0.01	< 0.01	< 0.01
SEM[2]					0.87	1.05	267	1.38	1.27	0.61	0.70
Significant ($P < 0.05$) contrasts[3]					1, 2	1, 2	-	1, 2	1, 2	1, 2	1, 2

[1]FOM = Apparently fermented OM

[2]For n = 6.

[3]1 = control vs average of adenine and adenosine; 2 = adenine vs adenosine.

[4]The thiamin x amprolium interaction was only significant for acetate ($P = 0.03$) and propionate ($P < 0.01$) molar percentages. The thiamin x intracellular synthesis inhibition was only significant ($P < 0.01$) for butyrate molar percentage. The amprolium x intracellular synthesis inhibition was only significant ($P < 0.01$) for acetate molar percentage. The three-way interaction was not significant for any dependent variable.

(Cotta, 1990). It is possible that adenosine stimulated the growth of those species in the present experiment.

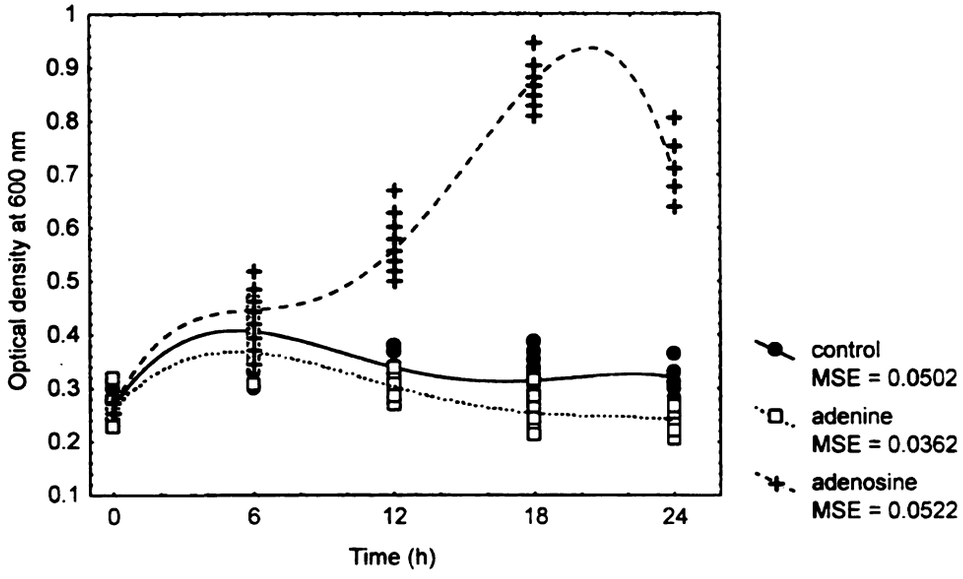

Figure 2-1. Effects of the addition adenine or adenosine on optical density of ruminal bacterial cells

Amprolium addition decreased ($P < 0.01$) CH_4 production by 42% (3.6 vs 6.2 μmol; Table 2-2). Surprisingly, the addition of thiamin also decreased ($P = 0.04$) CH_4 production by 22%. This result contradicts the hypothesis that thiamin would be used by ruminal microorganisms for pyruvate oxidative decarboxylation, a reaction that provides precursors for CH_4 formation. It agrees, though, with previous work in which thiamin addition decreased CH_4 production in continuous culture between 6 and 22% (Alves de Oliveira et al., 1996). However, in another study, there was no effect of thiamin addition on CH_4 production (Alves de Oliveira et al., 1997). Thiamin may have stimulated an

alternative H sink in the present experiment, therefore, decreasing methanogenesis. In agreement with Alves de Oliveira et al. (1997), thiamin addition did not have an effect on CO_2 release in the present experiment. Neither did amprolium, which would suggest that this structural analog of thiamin did not decrease CH_4 production by blocking pyruvate oxidative decarboxylation.

Adenine and adenosine decreased ($P < 0.01$) CH_4 production by 47 (4.1 vs 8.0 μmol) and 76% (2.7 vs 8.0 μmol), respectively. We are unaware of previous reports measuring the effects of nucleotides or nucleosides on CH_4 production in the rumen. We had hypothesized that the addition of adenine or adenosine would hinder thiamin intracellular synthesis. This would result in thiamin not being available to act as a cofactor in pyruvate oxidative decarboxylation, and the reaction would be blocked. Ultimately, this would decrease the availability of CO_2 and reducing equivalents for CH_4 production. Although both adenine and adenosine decrease CH_4 production, the addition of thiamin did not supress their effect on methanogenesis. This suggests that the decrease in methanogenesis caused by adenine and adenosine was unrelated to their hypothesized inhibition of thiamin intracellular synthesis.

Thiamin addition decreased ($P < 0.01$) propionate molar percentage. In agreement, Alves de Oliveira et al. (1996) found that the addition of thiamin to a ruminal continuous culture (with normal, but not with salts-reduced, artificial saliva) decreased propionate, and increased acetate molar percentage. Theoretically, one would expect this result, if thiamin was used as a cofactor of pyruvate oxidative decarboxylation. However, the addition of thiamin to the diet of sheep that served as donors of ruminal fluid increased propionate molar percentage in ruminal fermentation in vitro at the expense of

butyrate (Naga et al., 1975; Candau and Kone, 1980). Naga et al. (1975) considered that thiamin could have altered the VFA pattern directly, or indirectly by reducing ruminal motility and outflow in the thiamin deficient animals. Candau and Kone (1980) speculated that thiamin could act at the re-oxidation of intracellular cofactors, withdrawing reducing equivalents from methanogenesis and diverting them to the reduction of lactate to propionate. Alves de Oliveira et al. (1997) did not find an effect of thiamin addition on the VFA profile. The reasons for the discrepancies between experiments are unknown. In the present experiment, amprolium decreased ($P < 0.01$) acetate molar percentage, especially when thiamin was added. Adenosine, in contrast, prevented the decrease in acetate molar percentage caused by amprolium ($P = 0.03$). As adenosine itself strongly decreased ($P < 0.01$) acetate molar percentage, it is possible that both amprolium and adenosine acted on the same species of acetate producers, as their effects were not additive. In the absence of added thiamin, amprolium decreased ($P = 0.01$) propionate molar percentage. This was contrary to the experiment's hypothesis, for it was expected that an inhibition of pyruvate oxidative decarboxylation would divert the C in pyruvate from acetate and butyrate to propionate formation. However, when thiamin was present, amprolium increased ($P = 0.02$) propionate molar percentage. This interaction is difficult to interpret, and, considering also that the inhibition of CH_4 formation by amprolium was independent from thiamin addition, it is likely that amprolium did not decrease CH_4 production by inhibiting pyruvate oxidative decarboxylation.

Adenosine decreased ($P < 0.05$) butyrate molar percentage and greatly stimulated ($P < 0.05$) propionate. Adenine decreased ($P < 0.05$) butyrate molar percentage.

Thiamin addition decreased ($P < 0.01$) NH_4^+ concentrations (22.3 vs 20.1 mg/dL). This could be due to less fermentation of amino acids, or to an increase in microbial protein synthesis, as found by Candau and Kone (1980). In contrast, Naga et al. (1975) found a decrease in microbial growth as a result of adding thiamin. Alves de Oliveira et al. (1996, 1997) did not find any effect of thiamin addition upon microbial protein synthesis. Amprolium also decreased ($P = 0.01$) NH_4^+ concentration, while adenosine increased ($P < 0.05$) it. This could be explained by the deamination occurring in the catabolism of adenosine to hypoxanthine (Voet and Voet, 1995). The presence of the ribose moiety seemed to have been a requirement for deamination to occur, as the addition of adenine tended ($P = 0.08$) to decrease NH_4^+ concentrations.

The ratio of CO_2 to CH_4 in the triple control (thiamin, no amprolium, and no adenine or adenosine) was almost of 50 to 1, which is unusually large (Moss, 1993). Microbial growth in the control, as estimated through the increase in optical density, was almost non-existent (Figure 2-1). Some of the experimental procedures (washing the cells with a buffer, absence of ruminal fluid in the medium, use of very rapidly fermentable substrates) could have biased the microbial community that survived, and created an atypical fermentation. Amprolium, adenine, and adenosine decreased methanogenesis, although the mechanisms appeared to differ from the original hypothesis. In Experiments 2 and 3, therefore, the effects of adenine, adenosine, and amprolium, were studied under more classical in vitro procedures. As the mechanisms by which adenine and adenosine decreased CH_4 production in Experiment 1 were unrelated to the addition of amprolium, we studied them separately, adenine and adenosine in Experiment 2, and amprolium in Experiment 3.

Experiment 2

In Experiment 1, the strong inhibition of methanogenesis caused by adenine and adenosine was independent of thiamin addition. In Experiment 1, adenine and adenosine had opposite effects on fermented OM and microbial growth. As this could be caused by adenosine's ribose moiety, a treatment with ribose alone, and a treatment with adenine and ribose, were also included. Adenine, with or without ribose, did not affect CH_4 production (Table 2-3). The addition of ribose alone increased ($P = 0.03$), and of adenosine tended to increase ($P = 0.07$), methanogenesis. However, when CH_4 output was related to FOM, there were no differences among treatments.

The addition of ribose, either pure (control vs ribose, and adenine vs adenine + ribose) or as part of the adenosine molecule (adenine vs adenosine), always promoted ($P < 0.05$) OM fermentation (Table 2-3). On the contrary, adenine was inhibitory to fermentation. The addition of ribose, alone (control vs adenine + ribose, $P = 0.69$, Scheffé test), or as the ribose moiety in adenosine (control vs adenosine, $P = 0.58$) relieved the inhibition caused by adenine. If, as hypothesized, adenine caused an inhibition of thiamin synthesis, the reactions of the pentose phosphate pathway catalysed by transketolase could have been affected. However, this should not affect the supply of ribose, as ribose phosphate is a substrate, rather than a product, of these reactions (Voet and Voet, 1995). Therefore, the relief of adenine's fermentation inhibition by ribose would have been due to the use of the latter as an energy source. Although different ruminal microbial species differed in their ability to use ribose as an energy source, *Selenomonas ruminantium* strains could attain growth rates only slightly lower than with glucose (Cotta, 1990). The addition of adenine increased ($P < 0.01$) acetate molar percentage and decreased ($P <$

Table 2-3. Effects of adenine, adenosine and ribose on end products of ruminal in vitro fermentation (Exp. 2)

	Control	Adenine	Ribose	Adenine + ribose	Adenosine	SEM	P =	Significant ($P < 0.01$) planned contrasts[1]
CH_4, µmol	513	454	668	585	594	29.1	< 0.01[3]	3
CO_2, µmol	2302	2243	2477	2432	2443	36.0	< 0.01[3]	3
Total VFA, mM	87.3	86.3	103	100	102	0.47	< 0.01	2, 3
Acetate, µmol	2274	2281	2834	2736	2756	35.4	< 0.01	2, 3
Propionate, µmol	706	633	828	818	842	10.5	< 0.01	1, 2, 3
Butyrate, µmol	223	208	320	296	307	8.80	< 0.01	2, 3
FOM[2], %	52.8	46.6	58.8	51.2	53.3	0.72	< 0.01	1, 3, 4
CH_4/FOM, µmol/mg	1.76	1.57	1.81	1.64	1.62	0.08	0.30	none
NH_4^+, mg/dL	38.9	52.8	33.9	48.7	62.5	1.67	< 0.01	1, 2, 3, 4, 5

[1]1 = control vs adenine; 2 = control vs adenosine; 3 = adenine vs adenosine; 4 = ribose vs adenosine; 5 = adenine + ribose vs adenosine.

[2]FOM = Apparently fermented OM

[3]The Kruskal - Wallis test was performed because of heterogeneity of variances (Levene test on absolute deviations $P < 0.05$).

0.01) propionate, while adenosine decreased ($P < 0.01$) acetate and increased ($P < 0.01$) butyrate. Ribose increased ($P < 0.01$) butyrate molar percentage.

It was shown that 62% of added adenine (approximately 1.2 mM) was degraded after 4 h of incubation in ruminal fluid (McAllan and Smith, 1973). Hypoxanthine and xanthine accounted for 33 and 7%, respectively, of the adenine initially present. All of the added adenosine (approximately 1.2 mM) was degraded within 1 h, and 78% of its initial concentration was recovered as inosine, which was in turn converted to hypoxanthine. Therefore, deamination seemed to proceed faster when the ribose moiety was present. In Experiment 1, adenosine, but not adenine, increased NH_4^+ concentrations. In Experiment 2, the addition of adenine, with ($P = 0.02$) or without ribose ($P < 0.01$), and of adenosine ($P < 0.01$), increased NH_4^+ concentrations; however, NH_4^+ was higher ($P < 0.01$) with adenosine than with adenine. Ruminal protozoa have been shown to catabolize adenine to xanthine and hypoxanthine (Coleman and Laurie, 1974, 1977); however, we are not aware of reports on their use of adenosine. In Experiment 1, the low speed centrifugation for isolating the cell pellet must have removed the protozoa. If protozoa metabolize more adenine in relation to adenosine than bacteria, that could explain why adenine increased NH_4^+ concentrations in Experiment 2 but not in Experiment 1. Also, Cotta (1990) found that ruminal bacteria differed in their ability to use nucleosides, N bases, and ribose. It is then possible that the procedures used in Experiment 1 biased the microbial population so as to decrease the catabolism of adenine.

The effects of adenine and adenosine on fermentation were quite different from Experiment 1. A washed cell suspension was used in Experiment 1, whereas, a crude ruminal fluid and buffer mixture was used in Experiment 2. Differences in the microbial

species present must have existed between the two experiments. The ratio of CO_2 to CH_4 in Experiment 2 was more typical of a ruminal fermentation (Moss, 1993) . Therefore, it is concluded that Experiment 1 results did not represent a typical ruminal fermentation.

Experiment 3

The depression in CH_4 production caused by amprolium in Experiment 1 was independent of the addition of either thiamin, adenine, or adenosine. Hence, the effects of amprolium and another thiamin structural analog, oxythiamin, were studied in batch fermentation with ruminal fluid. Methane production was decreased ($P < 0.05$) by 23 (244 vs 315 μmol) and 8% (289 vs 315 μmoles) by oxythiamin and amprolium at 5 mM, respectively (Table 2-4). Increasing the concentrations to 10 mM did not decrease methanogenesis further. The effects of amprolium on CH_4 production were due to less fermentation, as CH_4 production per milligram of FOM did not change. However, oxythiamin addition decreased ($P < 0.01$) CH_4 production per milligram of FOM by about 17% at both concentrations. As H_2 concentration did not increase, a direct effect on methanogens seems unlikely. Oxythiamin decreased ($P < 0.01$) the molar percentage of acetate and butyrate and increased ($P < 0.01$) propionate. This would agree with an inhibition of pyruvate oxidative decarboxylation; however, there is not evidence that this was the mechanism by which methanogenesis was decreased. Increases in propionate molar percentage and decreases in acetate, when methanogenesis is inhibited, have been previously reported (Russell and Martin, 1984; Martin and Macy, 1985; García-López et al., 1996). The changes in VFA profile may be a consequence of the decrease in methanogenesis rather than the latter been caused by the increase in propionate.

Table 2-4. Effect of thiamin structural analogs on ruminal in vitro fermentation (Exp. 3)

	Control	Oxy[1] 5 mM	Oxy[1] 10 mM	Amp[1] 5 mM	Amp[1] 10 mM	SEM	P =	Significant contrasts[2]
CH_4, µmol	315	244	239	289	279	5.8	< 0.01	1, 2, 3
CO_2, µmol	646	647	643	674	705	30.7	0.58[4]	None
H_2, µmol	0.43	0.46	0.49	0.60	0.48	0.07	0.58	None
Total VFA, mM	48.9	47.7	47.1	46.9	45.8	0.28	0.02[4]	1, 3
Acetate, µmol	814	713	697	744	724	20.3	< 0.01[4]	1, 3
Propionate, µmol	298	353	348	280	251	5.3	< 0.01	1, 2, 3
Butyrate, µmol	74.3	58.8	56.6	61.3	55.6	3.66	< 0.01	1, 2, 3
FOM[3], %	56.7	53.2	51.5	51.4	48.2	1.50	0.02	1, 3
CH_4/FOM, µmol/mg	2.99	2.48	2.49	3.07	3.12	0.012	< 0.01	1
pH	7.3	7.2	7.1	7.2	7.2	0.07	0.18	1
NH_4^+, mg/dL	30.7	27.9	24.8	29.3	28.2	0.60	< 0.01	1, 3

[1]Oxy = oxythiamin; Amp = amprolium.

[2]1 = significant ($P < 0.05$) linear relationship for oxythiamin; 2 = significant ($P < 0.05$) quadratic relationship for oxythiamin; 3 = significant ($P < 0.05$) linear relationship for amprolium; 4 = significant ($P < 0.05$) quadratic relationship for amprolium.

[3]FOM = Apparently fermented OM

[4]The Kruskal - Wallis test was done due to heterogeneity of variances (Levene test on absolute deviations $P < 0.05$).

Amprolium increased ($P < 0.01$) acetate and decreased butyrate ($P = 0.02$) molar percentage. It was previously found that amprolium decreased propionate molar percentage, without affecting the rest of the VFA profile (Horton and Stockdale, 1979). A decrease in propionate, and an increase in butyrate, were reported when amprolium was added to ruminal continuous cultures (Heitmann and Yehya Taka, 1970-1971). Results from those studies the current experiment are contrary to the hypothesis that amprolium would impair thiamin utilization for thiamin oxidative decarboxylation, resulting in pyruvate carbon being diverted from acetate and butyrate towards propionate.

An antimethanogenic strategy based on the use of oxythiamin would require the delivery of ruminal-protected thiamin together with oxythiamin, so as to avoid potential toxic effects of the latter on the host animal. Given the small amounts that methanogenesis was decreased by oxythiamin, further work in this line is not recommended.

Experiment 4

None of the additives had any effect on CH_4 or CO_2 production (Table 2-5). Disappearance of all four additives was complete (data not shown). Added pyruvate, bromopyruvate or fluoropyruvate did not change the VFA molar proportions. This suggests that bromopyruvate and fluoropyruvate were dehalogenated, converted into pyruvate and metabolized. Hydroxypyruvate, however, shifted ($P < 0.05$) the VFA molar proportions towards butyrate at the expense of acetate, which may indicate that part of this additive was catabolized by an alternative pathway. Interestingly, hydroxypyruvate increased total OM (substrate + additive) and the alfalfa substrate fermentation by 6.4 ($P < 0.01$) and 5.3 ($P = 0.02$) percentage units, respectively. As total CH_4 production was not affected, CH_4 per milligram of FOM tended ($P = 0.05$) to be 16% lower than in the control.

The pyruvate derivatives did not inhibit CH_4 production, and, except for hydroxypyruvate, did not alter the VFA profile. This indicates that they did not inhibit pyruvate oxidoreductases as it was hypothesized. As they were totally metabolized, they must have been taken up by the cells. They might have been metabolized before they

Table 2-5 Effect of pyruvate derivatives on end products of in vitro ruminal fermentation (Exp. 4)

	Control	Pyruvate	Br-pyruvate	F-pyruvate	HO-pyruvate	SEM	$P =$	Significant ($P < 0.0083$) planned contrasts[1]
CH_4, µmol	467	457	423	423	453	31.4	0.20	none
CO_2, µmol	1041	1054	1019	1090	1080	156	0.60[3]	none
H_2, µmol	0.4	0.4	0.3	0.8	0.4	0.065	< 0.01	4, 6
Total VFA, mM	51.0	52.9	51.4	50.9	55.2	0.25	0.01[3]	5, 6
Acetate, µmol	1244	1329	1260	1249	1336	17.7	0.02[3]	1, 5, 6
Propionate, µmol	335	350	334	338	382	5.5	< 0.01	5, 6
Butyrate, µmol	136	135	135	127	194	0.27	0.04[3]	5, 6
FOM[2], %	57.8	58.8	56.5	55.4	64.2	5.29	0.01[3]	5, 6
Fermented substrate, %	57.8	57.5	55.1	54.2	63.1	0.49	< 0.01[3]	5, 6
CH_4/FOM, µmol/mg	2.90	2.70	2.60	2.67	2.44	0.009	< 0.05	2
pH	6.8	6.8	6.8	6.8	6.7	0.29	0.51	none
NH_4^+, mg/dL	15.1	16.9	16.2	16.1	15.5	0.23	0.10[3]	none

[1]1 = control vs pyruvate; 2 = control vs average pyruvate derivatives; 3 = pyruvate vs average pyruvate derivatives; 4 = bromopyruvate vs Na-fluoropyruvate; 5 = bromopyruvate vs hydroxypyruvate; 6 = Na-fluoropyruvate vs hydroxypyruvate.

[2]FOM = Apparently fermented OM

[3]The Kruskal - Wallis test was performed because of heterogeneity of variances (Levene test on absolute deviations $P < 0.05$).

could inhibit pyruvate oxidoreductases, or pyruvate oxidoreductases structures of ruminal microorganisms could be different from the ones previously reported.

Implications

Pyruvate oxidative decarboxylation in ruminal fluid could not be inhibited, either directly, or through the inhibition of thiamin utilization. It is not known if the additives were taken up by ruminal microorganisms, and, if they were, why the intracellular effects hypothesized did not occur. If further work in this line is to be considered, basic research on thiamin uptake, synthesis and utilization, and on the enzymology of pyruvate oxidoreductases of different ruminal microorganisms would be needed.

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CHAPTER 3

Use of some novel alternative electron sinks to inhibit ruminal methanogenesis

Abstract

Several compounds were evaluated in vitro as alternative electron sinks to ruminal methanogenesis. They were incubated with ruminal fluid, buffer mixture, and finely ground alfalfa hay for 24 h, at 0, 6, 12, and 18 mM initial concentrations. The propionate enhancer oxaloacetic acid, the butyrate enhancer ß-hydroxybutyrate, and the butyrate unsaturated analog 3-butenoic acid were ineffective in decreasing methanogenesis. Nevertheless, ß-hydroxybutyrate increased the apparent OM fermentability of the alfalfa hay substrate from 58.0 to 63.4%, and 3-butenoic acid seemed to increase it from 62.0 to 73.7%. Almost all of added oxaloacetic acid and most of acetoacetate disappeared during the incubation, while only between 30.3 and 53.4% of ß-hydroxybutyrate disappeared. The butyrate enhancers acetoacetate and crotonic acid, and the butyrate unsaturated analog 2-butynoic acid, decreased methanogenesis by a maximum of 18, 9 and 9%, respectively. Crotonic acid at 18 mM initial concentration seemed to increase the substrate apparent OM fermentability from 57.0 to 68.2%. Between 78.6 and 100% of acetoacetate disappeared during the incubation. The propionate unsaturated analog propynoic acid, and the unsaturated ester ethyl 2-butynoate, decreased methanogenesis by a maximum of 76 and 79%, respectively. Less than 5% of propynoic acid disappeared. The substrate apparent fermentability was decreased by propynoic acid from 62.0 to 57.4%, and seemed to have been decreased by ethyl 2-butynoate from 62.0 to 29.3%.

More accurate measurements of the disappearance of some of the compounds studied are needed to better understand how they are metabolized and how they affect fermentation.

Introduction

Methane emission is an energy loss for ruminants, and also causes global warming (Moss, 1993). It would be beneficial both for the efficiency of production and the environment to divert reducing equivalents from ruminal methanogenesis into alternative electron sinks with a nutritional value for the host animal (Schulman and Valentino, 1976), such as propionate (Callaway and Martin, 1996).

Intermediates of the fermentation pathways that lead to propionate ("propionate enhancers") have been studied as alternative electron sinks to ruminal methanogenesis. Compounds that accept one pair of electrons in their conversion into propionate include malate (Martin and Streeter, 1995; Callaway and Martin, 1996; López et al., 1999), fumarate (Callaway and Martin, 1996), lactate, and acrylate (López et al., 1999). Oxaloacetate, however, accepts two pairs of electrons, and, theoretically, should be more effective in competing with methanogenesis as an alternative electron sink. To our knowledge, oxaloacetate has not been examined for this purpose.

Likewise, intermediates in the conversion of pyruvate into butyrate ("butyrate enhancers") also accept reducing equivalents (Miller and Jenesel, 1979). Butyrate enhancers have not been studied as alternative electron sinks to ruminal methanogenesis. Also, unsaturated analogs of propionate and butyrate with double and triple bonds could be reduced to these VFA, redirecting reducing equivalents away from CH_4 formation. These compounds, which are not normal intermediates of ruminal fermentation (except

for acrylate and crotonate), have not been studied as alternative electron sinks to ruminal methanogenesis.

The objective of this study was to evaluate the effects of oxaloacetate, butyrate enhancers, and unsaturated organic acids and esters on in vitro fermentation by mixed ruminal microbial cultures. It was hypothesized that the addition of these compounds would decrease CH_4 production in vitro by utilizing reducing equivalents.

Materials and Methods

Additives and concentrations

The intermediate of the propionate pathway, oxaloacetic acid [free acid, Sigma O 4126]; three intermediates of the butyrate pathway (Miller and Jenesel, 1979), acetoacetate [Li salt, Sigma A 8509], ß-hydroxybutyrate [Na salt, Sigma H 6501], and crotonic acid [free acid, Sigma C 4630]; the unsaturated propionate analog propynoic acid [free acid, Acros 13150-0100]; the unsaturated butyrate analogues 3-butenoic acid [free acid, Acros 15883-0250], and 2-butynoic acid [free acid, Acros 30806-0010]; and the unsaturated ester ethyl 2-butynoate [Aldrich 4341-76-8] were examined as alternative electron sinks to ruminal methanogenesis in vitro. Each of the additives, except for ethyl 2-butynoate, was added to Wheaton bottles as 1 mL aqueous solutions, so as to achieve 6, 12 and 18 mM initial concentrations, respectively. The hydrophobic ester, ethyl 2-butynoate, was added directly as a liquid (35.7, 71.3, and 107.0 µL, to achieve 6, 12 and 18 mM initial concentrations, respectively) together with 1 mL of deionized water. Controls received 1 mL of deionized water. The initial concentrations, which could be considered as relatively high, were chosen based on the additives hypothesized mode of

action: the effectiveness of an additive for withdrawing electrons from methanogenesis should be stoichiometrically related to the amount of additive reduced. As this was the first time these compounds were studied, a wide range of initial concentrations was chosen. Similar ranges of initial concentrations have been used in other studies that evaluated alternative electron sinks to ruminal methanogenesis (Martin and Streeter, 1995; Callaway and Martin, 1996; López et al., 1999).

Oxaloacetic acid, acetoacetate, ß-hydroxybutyrate, and crotonic acid were examined together in two experimental runs, while propynoic acid, 3-butenoic acid, 2-butynoic acid, and ethyl 2-butynoate were examined in the third and fourth experimental runs.

Ruminal fluid collection and incubation

Ruminal fluid was withdrawn two hours after the morning feeding from two mature Holstein cows fed alfalfa hay. It was mixed together, and strained through two layers of cheesecloth. It was then blended for 15 s under CO_2, and again strained through two layers of cheesecloth. One volume part of ruminal fluid was mixed with four volume parts of a bicarbonate and phosphate buffer (Goering and Van Soest, 1975), and 50 mL of the ruminal fluid and buffer mixture anaerobically delivered into 125-mL Wheaton bottles. All the bottles contained 300 mg of ground (0.2 mm screen mesh) alfalfa hay (11.4% CP in the DM) as substrate. Three samples of the ruminal fluid and buffer mixture were frozen for subsequent determination of VFA initial concentrations. Bottles were sealed under an O_2-free CO_2 atmosphere, and incubated in a shaking water bath at 39 °C for 24 h. At the end of the incubation, bottles were allowed to cool to room

temperature, and total gas production volume was measured (Callaway and Martin, 1996). Fermentation was then stopped by adding 1 mL of a 10% phenol solution.

Analytical procedures

Methane and CO_2 were analyzed (Callaway and Martin, 1996), using a Gow Mac series 750 flame ionization detector gas chromatograph (Gow Mac Instruments Co., Bridgewater, NJ) equipped with a 4' x 1/4" DC 200 stainless steel column (150 °C, carrier gas was N_2 at 820 kPa). A RGD2 Reduction Gas Detector (Trace Analytical, Menlo Park, CA), equipped with the same type of column, was used for H_2 analysis. The volume of gas produced was expressed as μmoles at 25 °C and 1 atm. A 5-mL aliquot was centrifuged (26,000 x *g*, 4 °C, 30 min), and the pH was measured in the supernatants (Digital Benchtop pH Meter, Cole-Parmer Instrument Company, Vernon Hills, IL). Volatile fatty acids, lactate, formate, ethanol, and the chemical additives were quantified by differential refractometry with a Waters HPLC (Waters Associates Inc., Milford, MA) equipped with a BioRad HPX 87H column (BioRad Laboratories, Hercules, CA). Separation was done by ion moderated partition. Solvent was 0.005 M H_2SO_4 at 0.6 $mL \cdot min^{-1}$. Column temperature was 65 °C. Sample injection volume was 15 μL. Ammonia was analyzed as reported before (Chaney and Marbach, 1962).

Calculations

Based on known biochemical pathways, some of the fermentation intermediates added were not expected to produce gases. Consequently, calculations based on VFA production stoichiometry (Marty and Demeyer, 1973) would have then overestimated apparently fermented OM (FOM). Therefore, FOM and substrate apparently fermented were calculated by mass balance from the net production of VFA, lactate, gases, and ammonia. As ethanol, formate, and succinate accumulated in some of the treatments, they were also included in the calculation:

FOM (%) = (gases + VFA + lactate + ethanol + formate + succinate + NH_4^+) x 100 /(substrate OM + additive OM), with all fermentation products produced, substrate and additives expressed in grams.

Substrate apparently fermented (%) =
(FOM (g) - additive disappeared during fermentation (g)) x 100 /(substrate OM (g))

Crotonic acid and 2-butynoic acid co-eluted off the HPLC column with isovalerate and isobutyrate, respectively. As the amounts of isovalerate and isobutyrate produced are relatively minor in comparison to acetate, propionate, and butyrate, isovalerate was not included in the calculations for estimating FOM in the crotonic acid treatments, and isobutyrate in the 2-butynoic acid treatments. Disappearance of crotonic acid and 2-butynoic acid are not reported. Similarly, disappearances are not reported for 3-butenoic acid and ethyl 2-butynoate, as these additives co-eluted off the HPLC column

with propionate and butyrate, respectively. Organic matter and substrate fermentation are not reported for these additives.

Hydrogen balances were calculated (Marty and Demeyer, 1973), with net production of ammonia (one mole of ammonia produced releases one mole of reducing equivalent pairs) also considered. Net production of ethanol, lactate, and formate were also considered, ethanol and lactate formations releasing and accepting one pair of reducing equivalents each (Voet and Voet, 1995), and formate incorporating one pair of reducing equivalents (Russell and Wallace, 1997):

H produced (μmoles) = $2A + P + 4B + 3V + NH_4^+ + E + L$

H incorporated (μmoles) = $2P + 2B + 4V + 4CH_4 + H_2 + F + E + L$

H recovery (%) = H incorporated x 100 / H produced

where A = acetate, P = propionate, B = butyrate, V = valerate, E = ethanol, L = lactate, and F = formate, all expressed as μmoles. VFA and lactate were considered as nutritionally useful H sinks, while methane, dihydrogen, formate, and ethanol were considered as H sinks without a nutritional value. The H balance was not calculated for 3-butenoic acid and ethyl 3-butynoate, as these additives co-eluted off the HPLC column with propionate and butyrate.

Statistical analysis

Two replicates per compound and concentration were used in each of the two experimental runs. The experimental run was modeled as a random block (Neter et al.,

1996): observation = overall mean + additive concentration + run + residual. Orthogonal contrasts were performed to determine linear, quadratic, and cubic effects of concentration. Significance was declared at $P < 0.05$.

Results

Oxaloacetic acid

Production of CH_4 linearly increased ($P < 0.01$) by 7, 8, and 13%, at 6, 12, and 18 mM initial concentration of oxaloacetic acid, respectively (Table 3-1). The release of CO_2 was linearly increased ($P < 0.01$). H_2 accumulation was similar to control.

Oxaloacetic acid was almost totally fermented. There was a linear increase in total VFA concentration ($P < 0.01$), and acetate ($P < 0.01$), propionate ($P = 0.01$), butyrate ($P < 0.01$), valerate ($P = 0.01$), and isovalerate ($P < 0.05$) production. Production of isobutyrate, final pH, and NH_4^+ concentration were not affected. Oxaloacetic acid linearly decreased ($P < 0.01$) the alfalfa substrate apparent fermentability from 58.0 to 35.8%. As the amount of FOM increased due to the additive disappearance, CH_4 production per milligram of FOM was decreased ($P < 0.01$) by oxaloacetic acid.

Table 3-1. Effects of the addition of oxaloacetic acid on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, µmol	421	452	454	476	< 0.01	0.46	0.13	5.65
CO_2, µmol	911	1091	1276	1327	< 0.01	0.37	0.98	40.1
H_2, µmol	0.41	0.46	0.44	0.61	0.01	0.36	0.47	0.038
Additive disappearance, µmol	-	302	606	912	< 0.01	0.87	-	5.25
Additive disappearance, %	-	98.7	99.0	99.3	0.64	0.98	-	0.896
Total VFA, mM	54.5	59.4	64.2	66.5	< 0.01	0.13	0.49	0.783
Total VFA production, µmol	1657	1917	2175	2296	< 0.01	0.13	0.49	41.5
Acetate, µmol	1111	1330	1547	1688	< 0.01	0.14	0.51	23.8
Propionate, µmol	345	365	377	372	0.01	0.09	0.81	6.67
Butyrate, µmol	136	151	165	172	< 0.01	0.51	0.75	5.84
Isobutyrate, µmol	16.9	17.1	26.8	5.40	0.34	0.08	0.14	5.47
Valerate, µmol	20.4	23.3	26.4	26.6	0.01	0.25	0.56	1.12
Isovalerate, µmol	27.8	30.7	32.9	33.6	< 0.05	0.89	0.82	0.071
FOM, %	58.0	58.2	54.3	55.2	0.28	0.88	0.43	2.43
Substrate fermentability[1], %	58.0	52.3	41.2	35.8	< 0.01	0.96	0.44	3.09
CH_4/FOM, µmol/mg	2.62	2.45	2.14	2.17	< 0.01	0.20	0.19	0.007
Final pH	6.86	6.99	6.79	6.94	0.92	0.87	0.10	0.080
NH_4^+, mg/dL	26.2	24.9	23.4	25.2	0.32	0.13	0.41	0.906
H produced, µmol	3224	3854	4401	4673	< 0.01	0.19	0.50	63.4
H incorporated, µmol	2719	2933	3040	3103	< 0.01	0.21	0.60	33.6
H recovery, %	81.8	76.2	68.5	66.5	< 0.01	0.02	0.03	0.40
Nutritionally useful H, %	38.4	38.3	40.2	38.4	0.48	0.24	0.12	0.36
Nutritionally non-useful H, %	61.6	61.7	59.8	61.6	0.48	0.24	0.12	0.36

[1]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g))

Oxaloacetic acid linearly increased ($P < 0.01$) both H produced and incorporated, but decreased ($P < 0.01$) H recovery from 81.8 to 66.5%. The percentage of nutritionally useful H incorporated was not affected by oxaloacetate (Table 3-1).

Acetoacetate

Addition of acetoacetate linearly decreased ($P = 0.03$) CH_4 production by 5, 18, and 10% at 6, 12, and 18 mM initial concentration, respectively (Table 3-2). Release of CO_2 was not affected, while H_2 accumulation was linearly decreased ($P < 0.01$) by 32%.

Acetoacetate co-eluted off our HPLC column with formate. As formate concentration in the rumen is normally very small (Hungate et al., 1970), reasonable disappearances could be calculated by assuming that there was no formate present. The percentage of acetoacetate disappeared decreased linearly ($P < 0.01$) with the initial concentration (Table 2-2). Total VFA concentration, and production of acetate, butyrate, and isovalerate were linearly increased ($P < 0.01$) by the addition of acetoacetate. Propionate, isobutyrate, and valerate production, the substrate OM apparent fermentability, and final pH were not affected. Methane produced per milligram of FOM was decreased ($P < 0.01$) as a result of the slight decrease in CH_4 production and the increase in the amount FOM due to the additive disappearance. Ammonia concentration was lowest ($P = 0.04$) at 12 mM initial concentration of acetoacetate.

Table 3-2. Effects of the addition of acetoacetate on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, μmol	421	400	346	377	0.03	0.13	0.12	15.2
CO_2, μmol	911	987	936	998	0.56	0.92	0.51	78.6
H_2, μmol	0.41	0.35	0.35	0.28	< 0.01	0.05	0.62	0.011
Additive disappearance, μmol	-	303	571	722	< 0.01	0.02	-	15.8
Additive disappearance, %	-	100	93.2	78.6	< 0.01	0.09	-	1.79
Total VFA, mM	54.5	63.1	70.9	75.8	< 0.01	0.12	0.68	1.08
Total VFA production, μmol	1657	2117	2530	2789	< 0.01	0.12	0.68	57.3
Acetate, μmol	1111	1542	1922	2110	< 0.01	0.03	0.51	45.7
Propionate, μmol	345	336	330	337	0.20	0.11	0.54	4.51
Butyrate, μmol	136	172	208	259	< 0.01	0.09	0.38	3.79
Isobutyrate, μmol	16.9	13.3	5.75	17.4	0.77	0.13	0.28	4.51
Valerate, μmol	20.4	20.2	23.7	20.0	0.70	0.20	0.08	1.24
Isovalerate, μmol	27.8	32.3	40.5	45.1	< 0.01	0.98	0.48	2.25
FOM, %	58.0	62.4	63.3	63.8	0.03	0.22	0.65	1.48
Substrate fermentability[1], %	58.0	58.3	56.6	58.8	0.93	0.55	0.41	1.53
CH_4/FOM, μmol/mg	2.62	2.10	1.61	1.60	< 0.01	< 0.01	0.20	0.007
Final pH	6.86	6.88	6.84	6.91	0.69	0.56	0.43	0.046
NH_4^+, mg/dL	26.2	24.8	23.7	25.3	0.41	0.04	0.42	0.680
H produced, μmol	3324	4326	5222	5817	< 0.01	0.11	0.71	80.9
H incorporated, μmol	2719	2699	2555	2782	0.88	0.09	0.13	42.6
H recovery, %	81.8	62.5	48.9	48.0	< 0.01	< 0.01	0.26	0.91
Nutritionally useful H, %	38.4	40.7	46.1	45.8	< 0.01	0.31	0.13	0.45
Nutritionally non-useful H, %	61.6	59.3	53.9	54.2	< 0.01	0.31	0.13	0.45

[1]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g))

Hydrogen produced was linearly ($P < 0.01$) increased by acetoacetate. As H was not affected, H recovery was decreased ($P < 0.01$) from 81.8 to 48.0%. The percentage of nutritionally useful H incorporated was maximal ($P = 0.02$) at 12 mM acetoacetate (Table 3-2).

β-Hydroxybutyrate

Addition of β-hydroxybutyrate did not affect CH_4 production or H_2 accumulation (Table 3-3). The release of CO_2 was linearly increased ($P = 0.03$). Similar to acetoacetate, β-hydroxybutyrate co-eluted off the HPLC column with formate. As with acetoacetate, it was assumed for calculating β-hydroxybutyrate disappearance that no formate was present. The percentage of β-hydroxybutyrate disappeared decreased linearly ($P < 0.01$) with its initial concentration. Total VFA concentration, and acetate and butyrate production were linearly increased ($P < 0.01$) by the addition of β-hydroxybutyrate. The substrate apparent fermentability was linearly increased ($P = 0.03$) from 58.0 to 63.4%. However, as not all the additive disappeared, FOM tended ($P = 0.07$) to decrease from 58.0 to 55.0%. Propionate, isobutyrate, valerate, and isovalerate production, the final pH, and NH_4^+ concentration, were not affected (Table 3-3). Methane produced per milligram of FOM was decreased ($P < 0.01$) as a consequence of the increase in the amount FOM due to the additive disappearance.

Table 3-3. Effects of the addition of β-hydroxybutyrate on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, μmol	421	425	423	442	0.24	0.52	0.61	10.9
CO_2, μmol	911	927	937	1041	0.03	0.22	0.52	33.0
H_2, μmol	0.41	0.39	0.50	0.43	0.28	0.36	0.05	0.029
Additive disappearance, μmol	-	164	237	278	< 0.01	0.49	-	18.3
Additive disappearance, %		53.4	38.8	30.3	< 0.01	0.30	-	2.23
Total VFA, mM	54.5	59.4	61.2	65.0	< 0.01	0.59	0.31	1.01
Total VFA production, μmol	1657	1917	2016	2215	< 0.01	0.65	0.39	53.3
Acetate, μmol	1111	1296	1379	1511	< 0.01	0.47	0.35	34.1
Propionate, μmol	345	348	337	353	0.59	0.36	0.16	5.89
Butyrate, μmol	136	207	242	281	< 0.01	0.17	0.44	10.6
Isobutyrate, μmol	16.9	14.5	10.1	15.2	0.74	0.56	0.68	6.10
Valerate, μmol	20.4	23.1	21.1	24.1	0.29	0.94	0.24	1.76
Isovalerate, μmol	27.8	28.2	25.9	31.1	0.51	0.35	0.37	2.44
FOM, %	58.0	57.9	54.8	55.0	0.07	0.90	0.33	1.35
Substrate fermentability[1], %	58.0	58.4	58.4	63.4	0.03	0.13	0.43	1.38
CH_4/FOM, μmol/mg	2.62	2.38	2.29	2.16	< 0.01	0.23	0.41	0.003
Final pH	6.86	6.84	6.85	6.93	0.27	0.24	0.81	0.038
NH_4^+, mg/dL	26.2	24.9	25.4	24.8	0.51	0.79	0.58	1.17
H produced, μmol	3324	3991	4288	4724	< 0.01	0.40	0.39	89.8
H incorporated, μmol	2719	2902	2937	3131	< 0.01	0.94	0.36	50.2
H recovery, %	81.8	72.6	68.5	66.5	< 0.01	0.01	0.47	0.55
Nutritionally useful H, %	38.4	41.4	42.3	43.6	< 0.01	0.10	0.31	0.35
Nutritionally non-useful H, %	61.6	58.6	57.7	56.4	< 0.01	0.10	0.31	0.35

[1]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g))

Hydrogen produced and incorporated were linearly increased ($P < 0.01$) by β-hydroxybutyrate. Hydrogen recovery was decreased ($P < 0.01$) from 81.8 to 66.5%. The percentage of H incorporated into nutritionally useful products was linearly increased ($P < 0.01$) by β-hydroxybutyrate from 38.4 to 43.6% (Table 3-3).

Crotonic acid

Production of CH_4 was 4, 9, and 2 % lower ($P < 0.05$) than the control at 6,12, and 18 mM initial concentration of crotonic acid, respectively (Table 3-4). The release of CO_2 was linearly increased ($P = 0.01$) by 24%, and H_2 accumulation was not affected. Crotonic acid disappearance was not estimated because it co-eluted off the HPLC column with isovalerate. Total VFA concentration ($P < 0.01$), and production of acetate ($P < 0.01$), butyrate ($P < 0.01$), isobutyrate ($P = 0.04$), and valerate ($P < 0.01$) were increased by crotonic acid (Table 3-4). Propionate production and NH_4^+ concentration were not affected. If control levels of isovalerate are assumed, crotonic acid increased ($P < 0.05$; cubic response) the substrate apparent fermentation from 57.0 to 68.2%. Methane produced per milligram of FOM was decreased ($P < 0.01$) as a result of the decrease in CH_4 production and the increase in the amount FOM due to the additive disappearance. Final pH was linearly decreased ($P < 0.01$). Hydrogen produced and incorporated were linearly increased ($P < 0.01$) by crotonic acid, but H recovery was linearly decreased ($P < 0.01$) from 81.8 to 56.7%. The percentage of H incorporated into nutritionally useful products was linearly increased ($P < 0.01$) from 38.4 to 49.1% (data not shown).

Table 3-4. Effects of the addition of crotonic acid on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, μmol	421	406	385	413	0.31	< 0.05	0.22	9.19
CO_2, μmol	911	1013	1007	1127	0.01	0.85	0.27	44.5
H_2, μmol	0.41	0.43	0.57	0.46	0.17	0.16	0.08	0.042
Additive disappearance, μmol	-	NA[4]	NA	NA	NA	NA	NA	NA
Additive disappearance, %	-	NA	NA	NA	NA	NA	NA	NA
Total VFA, mM[1]	53.7	59.0	61.2	73.5	< 0.01	< 0.01	0.02	0.95
Total VFA production, μmol[1]	1629	1911	2030	2682	< 0.01	< 0.01	0.02	50.3
Acetate, μmol	1111	1269	1368	1854	< 0.01	< 0.01	0.02	33.8
Propionate, μmol	345	351	335	337	0.23	0.76	0.22	6.59
Butyrate, μmol	136	255	302	457	< 0.01	0.13	< 0.01	10.2
Isobutyrate, μmol	16.9	11.2	2.22	7.39	0.04	0.14	0.27	3.31
Valerate, μmol	20.4	24.8	23.8	27.3	< 0.01	0.71	0.11	1.19
Isovalerate, μmol	27.8	NA	NA	NA	NA	NA	NA	NA
FOM, %[2]	57.0	60.0	57.6	67.2	< 0.01	0.05	0.02	1.40
Substrate fermentability[3], %	57.0	NA	NA	NA	NA	NA	NA	NA
CH_4/FOM, μmol/mg	2.62	2.24	2.03	1.74	< 0.01	0.55	0.47	0.007
Final pH	6.86	6.94	6.72	6.67	< 0.01	0.19	0.04	0.045
NH_4^+, mg/dL	26.2	25.2	24.9	24.9	0.43	0.69	0.95	1.01

[1]Isovalerate not included in the calculations.

[2]FOM = apparently fermented OM.

[3]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g))

[4]NA = not available. As crotonic acid co-eluted the HPLC column with isovalerate, their final concentrations, and hence substrate fermentation, could not be determined.

Propynoic acid

Methane production was decreased ($P < 0.01$) by 65, 72, and 76%, at 6, 12, and 18 mM initial concentration, respectively (Table 3-5). The release of CO_2 was linearly decreased ($P = 0.04$). Propynoic acid caused ($P < 0.01$) a 42, 53, and 51-fold increase in H_2 accumulation, at 6, 12, and 18 mM initial concentration, respectively.

Less than 5% of propynoic acid disappeared. Disappearance was similar for all initial concentrations of propynoic acid (Table 3-5). Total VFA concentration, acetate production, final pH, and NH_4^+ concentration were linearly decreased ($P < 0.01$). Propionate production was maximum at 6 mM initial concentration, and then decreased ($P < 0.01$). Butyrate production increased at 6 and 12 mM concentration of propynoic acid, and decreased at 18 mM ($P = 0.04$; quadratic response). Isobutyrate ($P = 0.02$; cubic response) and isovalerate ($P < 0.01$; cubic response) production were minimum at 6 mM initial concentration. Valerate production was not affected by propynoic acid concentration. The substrate apparent fermentability was decreased from 62.0 to 56.6 and 57.4% at 6 and 18 mM initial concentration, respectively, but not affected at 12 mM ($P < 0.01$; cubic response). Although FOM (%) was decreased ($P < 0.01$) by propynoic acid, CH_4 production per milligram of FOM was decreased by 63, 72, and 75%, at 6, 12, and 18 mM initial concentration, respectively.

Propynoic acid caused the accumulation of some unusual fermentation products (data not shown). Formate was increased ($P < 0.01$) from 0.48 to 5.59 mM, and ethanol from 0.11 to 3.17 mM, both at 12 mM propynoic acid. Also, succinate concentration was increased ($P < 0.01$) from 0.02 to 1.10 mM.

Table 3-5. Effects of the addition of propynoic acid on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, μmol	447	155	123	108	< 0.01	< 0.01	< 0.01	5.18
CO_2, μmol	1010	874	906	794	0.04	0.84	0.25	55.7
H_2, μmol	0.93	38.6	49.5	47.5	< 0.01	< 0.01	0.01	0.81
Additive disappearance, μmol	-	10.3	20.9	41.1	0.01	0.54	-	6.06
Additive disappearance, %	-	3.38	3.42	4.48	0.39	0.54	-	0.85
Total VFA, mM	56.8	52.5	51.5	49.3	< 0.01	0.13	0.14	0.62
Total VFA production, μmol	1784	1557	1503	1387	< 0.01	0.13	0.15	32.8
Acetate, μmol	1224	961	922	880	< 0.01	< 0.01	< 0.01	13.5
Propionate, μmol	342	391	335	290	< 0.01	< 0.01	< 0.01	7.23
Butyrate, μmol	144	152	155	135	0.35	0.04	0.50	5.74
Isobutyrate, μmol	18.7	8.53	25.2	22.7	0.11	0.31	0.02	3.53
Valerate, μmol	22.5	24.2	21.5	34.1	0.30	0.42	0.52	6.49
Isovalerate, μmol	32.4	20.8	44.2	25.6	0.85	0.33	< 0.01	3.31
FOM, %	62.0	52.3	53.7	45.7	< 0.01	0.45	< 0.01	1.04
Substrate fermentability[1], %	62.0	56.6	62.5	57.4	0.16	0.95	< 0.01	1.15
CH_4/FOM, μmol/mg	2.73	1.02	0.77	0.69	< 0.01	< 0.01	< 0.01	0.005
Final pH	7.01	6.94	6.83	6.80	< 0.01	0.63	0.46	0.033
NH_4^+, mg/dL	23.9	18.0	18.3	16.8	< 0.01	< 0.01	0.02	0.64
H produced, μmol	3562	3131	3138	2912	< 0.01	0.17	0.06	48.1
H incorporated, μmol	2860	2022	2099	1914	< 0.01	< 0.01	< 0.01	37.3
H recovery, %	81.2	65.0	67.6	66.0	< 0.01	< 0.01	< 0.01	0.45
Nutritionally useful H, %	36.7	57.8	50.6	50.2	< 0.01	< 0.01	< 0.01	0.55
Nutritionally non-useful H, %	62.3	42.2	49.4	49.8	< 0.01	< 0.01	< 0.01	0.55

[1]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g))

Propynoic acid decreased ($P < 0.01$) H produced and incorporated. Hydrogen recovery decreased ($P < 0.01$) from 81.2 to 66.0%. The percentage of H incorporated into nutritionally useful fermentation end products was increased ($P < 0.01$) from 36.7 to 49.8% (Table 3-5).

3-Butenoic acid

Methane production tended ($P = 0.07$) to decrease linearly by 5%, and H_2 accumulation was not affected (Table 3-6). There was a 25% linear increase ($P = 0.02$) in CO_2 release with the addition of 3-butenoic acid. Acetate and butyrate production were linearly ($P < 0.01$) increased. Valerate and isovalerate production were maximum ($P < 0.01$) at 6 mM initial concentration of 3-butenoic acid. Isobutyrate tended ($P = 0.07$) to increase linearly. The co-elution of 3-butenoic acid and propionate off the HPLC column prevented us from finding propionate production and 3-butenoic acid disappearance. If 100% disappearance of 3-butenoic acid is assumed, it would have increased ($P < 0.05$; cubic response; data not shown) the substrate apparent fermentability from 62.0 to 74.0, 68.3, and 73.7%, at 6, 12, and 18 mM initial concentration, respectively. Final pH tended ($P = 0.05$) to decrease linearly. Ammonia concentration was not affected.

Table 3-6. Effects of the addition of 3-butenoic acid on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, μmol	447	449	423	424	0.07	0.96	0.26	10.3
CO_2, μmol	1010	1075	1046	1264	0.02	0.23	0.23	59.2
H_2, μmol	0.93	0.99	0.77	3.53	0.12	0.20	0.48	0.97
Additive disappearance, μmol	-	NA[2]	NA	NA	NA	NA	NA	NA
Additive disappearance, %	-	NA	NA	NA	NA	NA	NA	NA
Total VFA, mM	56.8	NA	NA	NA	NA	NA	NA	NA
Total VFA production, μmol	1784	NA	NA	NA	NA	NA	NA	NA
Acetate, μmol	1224	1578	1923	2226	< 0.01	0.25	0.72	20.6
Propionate, μmol	342	NA	NA	NA	NA	NA	NA	NA
Butyrate, μmol	144	340	443	550	< 0.01	< 0.01	0.03	8.2
Isobutyrate, μmol	18.7	32.9	29.1	32.3	0.07	0.21	0.20	4.01
Valerate, μmol	22.5	91.0	60.7	58.3	< 0.01	0.01	< 0.01	4.63
Isovalerate, μmol	32.4	94.4	82.9	70.2	< 0.01	< 0.01	< 0.01	4.58
FOM, %	62.0	NA	NA	NA	NA	NA	NA	NA
Substrate fermentability[1], %	62.0	NA	NA	NA	NA	NA	NA	NA
Final pH	7.01	6.79	6.78	6.80	0.05	0.08	0.54	0.061
NH_4^+, mg/dL	23.9	21.4	22.6	22.0	0.20	0.22	0.12	6.97

[1]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g)).

[2]NA = not available. As 3-butenoic acid co-eluted the HPLC with propionate, their final concentrations, and total VFA, could not be determined, and fermentation could not be estimated.

2-Butynoic acid

Methane production was linearly decreased ($P < 0.01$) by 4, 6, and 9% at 6, 12, and 18 mM initial concentration, respectively (Table 3-7). The release of CO_2 and H_2 accumulation, were not affected. Total VFA concentration was maximum ($P < 0.01$) at 6 mM propynoic acid. Acetate and propionate production were decreased ($P < 0.01$) at 12 and 18 mM initial concentration. Butyrate, valerate, and isovalerate production were maximum ($P < 0.01$) at 6 mM initial concentration. Apparently FOM was decreased ($P < 0.01$) by 2-butynoic acid from 61.4 to 48.6%. Methane produced per milligram of FOM was unaffected. Final pH and NH_4^+ concentration were both linearly decreased ($P < 0.01$) by 2-butynoic acid. Hydrogen produced and incorporated were highest ($P < 0.01$) at 6 mM 2-butynoic acid, but H recovery was not affected. The percentage of H incorporated into nutritionally useful end products was highest ($P < 0.01$) at 6 mM 2-butynoic acid (data not shown).

Table 3-7. Effects of the addition of 2-butynoic acid on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, µmol	447	430	422	407	< 0.01	0.91	0.57	6.74
CO_2, µmol	1010	958	1109	1074	0.17	0.88	0.13	51.4
H_2, µmol	0.93	0.75	1.08	1.32	0.15	0.34	0.52	0.21
Additive disappearance, µmol	-	NA[4]	NA	NA	NA	NA	-	NA
Additive disappearance, %	-	NA	NA	NA	NA	NA	-	NA
Total VFA, mM[1]	56.2	60.9	55.9	54.7	< 0.01	< 0.01	< 0.01	0.34
Total VFA production, µmol[1]	1765	2016	1750	1686	< 0.01	< 0.01	< 0.01	18.3
Acetate, µmol	1224	1236	1144	1125	< 0.01	0.15	< 0.01	9.76
Propionate, µmol	342	354	311	296	< 0.01	0.01	< 0.01	4.18
Butyrate, µmol	144	207	165	162	0.62	< 0.01	< 0.01	4.61
Isobutyrate, µmol	18.7	NA	NA	NA	NA	NA	NA	NA
Valerate, µmol	22.5	129	74.4	59.0	0.01	< 0.01	< 0.01	3.85
Isovalerate, µmol	32.4	90.0	55.6	43.9	0.99	< 0.01	< 0.01	3.70
FOM[2], %	61.4	64.6	54.1	48.6	< 0.01	< 0.01	< 0.01	0.62
Substrate fermentation[3], %	61.4	NA	NA	NA	NA	NA	NA	NA
CH_4/FOM, µmol/mg	2.73	2.65	2.85	2.55	0.58	0.41	0.20	0.019
Final pH	7.01	7.03	6.88	6.78	< 0.01	0.22	0.27	0.044
NH_4^+, mg/dL	23.9	23.6	21.3	21.6	< 0.01	0.51	0.07	4.76

[1]Isobutyrate not included.

[2]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g))

[3]NA = not available. As 3-butenoic acid co-eluted the HPLC column with isobutyrate, their final concentrations could not be determined, and hence the substrate fermentation, could not be estimated.

Ethyl 2-butynoate

Methane production was linearly decreased ($P < 0.01$) by 24, 64, and 79%, at 6, 12, and 18 mM initial concentration, respectively (Table 3-8). Release of CO_2 was also linearly decreased ($P < 0.01$) by ethyl 2-butynoate. Ethyl 2-butynoate caused ($P < 0.01$) a 12, 28, and 37-fold increase in H_2 accumulation, at 6, 12, and 18 mM initial concentration, respectively. Acetate production and NH_4^+ concentration were linearly decreased ($P < 0.01$). Propionate, valerate, and isovalerate production were maximum at 6 mM initial concentration, and dropped at 12 and 18 mM ($P < 0.01$). Butyrate production was not determined because it co-eluted off the HPLC column with ethyl 2-butynoate. Isobutyrate production was linearly increased ($P < 0.01$) 7, 13, and 18-fold, at 6, 12, and 18 mM initial concentration, respectively. Apparent fermentation of OM and substrate were not estimated as the co-elution of ethyl 2-butynoate and butyrate off the HPLC column prevented the determination of butyrate production and ethyl 2-butynoate disappearance. If 100% disappearance is assumed, ethyl 2-butynoate would have linearly decreased ($P < 0.01$) substrate apparent fermentability from 62.0 to 29.3% (data not shown). Final pH was not affected.

Ethyl 2-butynoate caused the accumulation of some unusual end products of ruminal fermentation (data not shown). Formate concentration was increased ($P < 0.01$) from 0.48 to 6.11 mM at 18 mM ethyl 2-butynoate. Also, ethanol concentration was increased ($P < 0.01$) from 0.12 to 10.4 mM at 18 mM ethyl 2-butynoate.

Table 3-8. Effects of the addition of ethyl 2-butynoate on in vitro ruminal fermentation

	Initial concentration, mM				Linear effect $P =$	Quadratic effect $P =$	Cubic effect $P =$	SEM
	0	6	12	18				
CH_4, μmol	447	340	160	93.9	< 0.01	0.63	0.33	40.3
CO_2, μmol	1010	909	704	748	< 0.01	0.30	0.26	65.6
H_2, μmol	0.93	11.1	25.8	34.4	< 0.01	0.80	0.47	3.10
Additive disappearance, μmol	-	NA[2]	NA	NA	NA	NA	NA	NA
Additive disappearance, %	-	NA	NA	NA	NA	NA	NA	NA
Total VFA, mM	56.2	NA	NA	NA	NA	NA	NA	NA
Total VFA production, μmol	1765	NA	NA	NA	NA	NA	NA	NA
Acetate, μmol	1224	1181	931	792	< 0.01	0.32	0.16	45.5
Propionate, μmol	342	427	358	329	0.04	< 0.01	< 0.01	9.89
Butyrate, μmol	144	NA	NA	NA	NA	NA	NA	NA
Isobutyrate, μmol	18.6	123	245	340	< 0.01	0.90	0.78	36.2
Valerate, μmol	22.5	87.6	47.5	32.5	0.50	< 0.01	< 0.01	3.22
Isovalerate, μmol	32.4	72.8	59.7	56.0	0.02	< 0.01	0.01	4.32
FOM, %	62.0	NA	NA	NA	NA	NA	NA	NA
Substrate fermentability[1], %	62.0	NA	NA	NA	NA	NA	NA	NA
Final pH	7.01	6.97	6.91	6.87	0.17	0.93	0.92	0.071
NH_4^+, mg/dL	23.9	20.0	18.3	17.0	< 0.01	0.05	0.53	0.55

[1]Substrate apparently fermented (%) = (FOM (g) - additive disappeared (g)) x 100 / (substrate OM (g))

[2]NA = not available. As ethyl 2-butynoate co-eluted the HPLC with butyrate, their final concentrations, and total VFA, could not be determined, and fermentation could not be estimated.

Discussion

Oxaloacetate and butyrate enhancers

Acetate, followed by CO_2, seemed to be the major C sink of the metabolism of added oxaloacetic acid. Therefore, most of added oxaloacetic acid was not fermented to propionate, as was hypothesized, but perhaps decarboxylated to pyruvate, and subsequently decarboxylated again to acetate, releasing one pair of reducing equivalents. The increase observed in the release of CO_2 suggests that oxaloacetic acid in fact underwent decarboxylation. The slight increase in CH_4 production might have been a consequence of the release of reducing equivalents in the oxidative decarboxylation of pyruvate into acetate. Oxaloacetate, is , however, an intermediate of a ruminal fermentation pathway leading to propionate (Russell and Wallace, 1997). It was expected to be metabolized to propionate, rather than to acetate. It is possible that most of externally added oxaloacetic acid was taken up by microbial species whose main fermentation end .product is acetate, rather than propionate.

Acetate, rather than butyrate, as was hypothesized, also seem to have been the major C sink of added acetoacetate and β-hydroxybutyrate. More of the latter, however, seemed to be converted to butyrate. Acetoacetate could have been broken down into two moles of acetate, which agrees with the fact that CO_2 release did not increase. The existence of a preferred pathway towards acetate could have allowed the greater disappearance observed for acetoacetate as compared to β-hydroxybutyrate, as β-hydroxybutyrate would need to be oxidized to acetoacetate in order to be converted to acetate. Similar to oxaloacetic acid, it is possible that microbial species different from the

butyrate producers that normally metabolize these compounds took the externally added additives, and metabolized them to acetate.

More of the added crotonic acid was fermented to butyrate, as compared to the other additives, but acetate was still an important C sink. Similarly, the sewage anaerobic bacterium *Syntrophomonas wolfei* catabolized crotonate to acetate and smaller proportions of butyrate and caproate (Beaty and McInerney, 1987). Thus, the added organic acids did not seem to have been metabolized only by the pathways of which they are intermediates in ruminal fermentation.

Oxaloacetic acid and β-hydroxybutyrate did not inhibit CH_4 production. Acetoacetate caused a small decrease in CH_4 production, without inhibiting fermentation or causing the accumulation of end products of fermentation without a nutritional value. The small decrease in CH_4 production caused by crotonic acid could be partly due to the decrease in pH that it caused (Van Kessel and Russell, 1996), as it was added as a free acid. Furthermore, the fact that the pH was not measured at the CO_2 partial pressure present in the Wheaton bottles before opening them, probably resulted in some overestimation of the final pH, due to loss of dissolved CO_2, as this is in equilibrium with H_2CO_3 (Kohn and Dunlap, 1998). ß-Hydroxybutyrate at 18 mM initial concentration stimulated the substrate apparent fermentability. Crotonic acid seemed to have the same effect. Stimulation of fermentation of a high roughage substrate has been reported for pyruvate, lactate, fumarate, malate, 2-oxoglutarate and tartrate (López et al., 1999). Due to its low disappearance, ß-hydroxybutyrate did not affect OM apparent fermentability. Butyrate absorbed through the rumen and omasal walls is converted into ß-hydroxybutyrate, and used as an energy source (McDonald et al., 1995). Externally

added ß-hydroxybutyrate escaped from ruminal fermentation could be usable for the ruminant, if it could be absorbed as such.

All of the additives decreased H recovery. The inhibition of methanogenesis may have stimulated some H sinks that were not measured, like sulfate and nitrate reductions (Stewart et al., 1997), and fatty acids biohydrogenation and synthesis (Czerkawski, 1986). Acetoacetate, ß-hydroxybutyrate, and crotonic acid increased the percentage of H incorporated into nutritionally useful sinks. ß-Hydroxybutyrate did not inhibit CH_4 production, so fermentation of the additive itself must not have produced additional CH_4, or produced less CH_4, than the substrate fermentation.

Unsaturated organic acids and esters

A shift of the VFA profile from acetate to propionate when methanogenesis is inhibited has been previously reported (Marty and Demeyer, 1973; García-López et al., 1996; Nagaraja et al., 1997). However, the acetate to propionate ratio decreased from 3.56 to 2.44 at 6 mM propynoic acid, and then increased to 2.74 and 3.05 at 12 and 18 mM, respectively ($P < 0.001$; quadratic response; data not shown). Ethyl 2-butynoate linearly decreased ($P < 0.001$) the acetate to propionate ratio from 3.56 at 0 mM to 2.75, 2.56, and 2.38, at 6, 12, and 18 mM initial concentration, respectively (data not shown).

Some unusual end products of fermentation accumulated when methanogenesis was inhibited by propynoic acid or ethyl 2-butynoate. Under normal conditions, methanogenesis keeps a low partial pressure of H_2 in the rumen (Wolin et al., 1997). Hydrogen accumulation has been observed with other methanogenesis inhibitors, like 2-bromoethanesulfonate (Martin and Macy, 1985) and 9,10-anthraquinone (García-López et

al., 1996). Inhibition of methanogenesis using 2-bromoethanesulfonate has also caused formate accumulation, because the increase in H_2 partial pressure displaces the equilibrium from HCO_3^- and H_2 towards formate formation (Wu et al., 1993). It is also possible that the inhibition of methanogenesis stimulated the disposal of reducing equivalents from pyruvate oxidative decarboxylation to acetyl-CoA into formate, a reaction catalyzed by formate lyases (Gottschalk and Andressen, 1979) instead of pyruvate oxidoreductases (Uyeda and Rabinowitz, 1971). Reducing equivalents spared from methanogenesis would also have been used to reduce acetyl-CoA to ethanol, as happens in pure cultures of *Ruminococcus albus* and *Neocallimastix frontalis* in the absence of methanogens (Wolin et al., 1997). The accumulation of H_2, formate, and ethanol indicates that the electrons not captured by methanogenesis were not efficiently disposed of into other alternative pathways such as propionate formation, or fatty acids synthesis and biohydrogenation (Czerkawski, 1986).

Succinate is a fermentation intermediate that normally does not accumulate in the rumen or mixed ruminal cultures, as it is converted to propionate by succinate utilizers (Wolin et al., 1997). It is interesting that the greatest accumulation of succinate occurred at 12 and 18 mM initial concentration of propynoic acid (0.85 and 1.10 mM, respectively), while propionate production was maximum at 6 mM. It is possible that succinate utilizers could have been overwhelmed by the amount of succinate formed at 12 and 18 mM propynoic acid. Added succinate at 34 mM initial concentration was metabolized to both acetate and propionate, although disappearance was not reported (Czerkawski and Breckenridge, 1972). There might be opportunities to increase the amounts of propionate formed by adding succinate utilizers to the fermentations, or by

stimulating the ones already present. Alternatively, the fact that propionate production decreased, rather than remained constant, at 12 and 18 mM propynoic acid, suggests a direct inhibition by propynoic acid on succinate utilizers. In support of this, it was found that added succinate at 29.7 mM was completely consumed by a mixed ruminal culture, and that more than 90% of it was decarboxylated to propionate (Samuelov et al., 1999). The initial concentration of succinate of 29.7 mM was much greater that the accumulation herein observed, yet succinate utilization was not overwhelmed in that study.

Despite the formation of unusual fermentation end products, propynoic acid increased the percentage of H incorporated into products nutritionally useful for the host animal. However, due to the decrease in H produced and to the formation of non-useful H sinks, H spared from methanogenesis by propynoic acid did not cause an increase in the absolute amount of H incorporated into useful sinks. Between two thirds and four fifths of the electrons released by fermentation were accounted by measured sinks. Little propynoic acid was metabolized, so it could have not acted as an electron sink itself. A H balance was not calculated for ethyl 2-butynoate because its co-elution with butyrate from the HPLC column prevented the determination of butyrate production.

It was hypothesized that propynoic acid and ethyl 2-butynoate would inhibit CH_4 production by being alternative electron sinks. However, it is doubtful that methanogenesis was inhibited based on electron withdrawal from the medium since: 1) accumulation of unusual, reduced end products like H_2, formate, and ethanol, was observed, and 2) almost all of the propynoic acid remained after 24 h incubation. Therefore, it was not hydrogenated to propionate or acrylate.

Propynoic acid decreased total OM apparent fermentability partly because the additive itself was not fermented. Apparent fermentability of the alfalfa substrate was decreased at 6 and 18 mM, but unaffected at 12 mM initial concentration. However, a higher proportion of the fermented products were nutritionally non-usable at 12 mM , compared to 6 mM initial concentration. As most of the inhibition of methanogenesis was already achieved at 6 mM initial concentration, the utilization of lower initial concentrations could be a way of minimizing the negative effects of propynoic acid on fermentation. This would decrease the proportion of the OM that is not fermented.

The inhibition of methanogenesis caused by 3-butenoic acid was small, but it might have stimulated substrate fermentation. Acetate, followed by butyrate, seemed to be the most important C sink for this additive.

2-Butynoic acid also caused small decreases in CH_4 production. Fermentation was inhibited at 12 and 18 mM initial concentration, but not at 6 mM. At 6 mM initial concentration, most 2-butynoic acid seemed to be metabolized to butyrate, valerate, and isovalerate. Disappearance of 2-butynoic acid could not be measured as it co-eluted from the HPLC column with isobutyrate; however, as changes in total VFA production were relatively small at 12 and 18 mM compared to the control, it is possible that most of 2-butynoic acid was not metabolized at those initial concentrations.

Implications

Propynoic acid and ethyl 2-butynoate decreased ruminal methanogenesis in vitro. Propynoic acid had some adverse effects on the substrate apparent fermentability, and ethyl 2-butynoate also seemed to be inhibitory to fermentation, although its disappearance

could not be measured. Both propynoic acid and ethyl 2-butynoate caused the formation of products without nutritional value. It is possible that organic acids that seemed to benefit fermentation, like β-hydroxybutyrate, crotonic acid, or 3-butenoic acid, could be fed to ruminants together with propynoic acid or ethyl 2-butynoate to relieve the negative effects on fermentation caused by the inhibitors of methanogenesis. Propynoic acid oral LD_{50} to rodents is 100 mg/kg (CCOHS), although its toxicity to ruminants at the doses inhibitory to ruminal methanogenesis would need to be evaluated. We are not aware of toxicity trials with ethyl 2-butynoate or 3-butenoic acid. Crotonic acid LD_{50} to rodents is between 1 and 4.8 g/kg (CCOHS). It might be less toxic to ruminants as it is a naturally occurring intermediate in ruminal fermentation (Miller and Jenesel, 1979). Likewise, acetoacetate and ß-hydroxybutyrate may be mildly toxic to ruminants for the same reason. Accurate measurements of the disappearances of some of the compounds studied are needed to fully understand what happened to, and as a consequence of, the addition of these chemicals. Their toxicity to ruminants, as well as the potential hazards for humans and the environment, would also need to be assessed.

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CHAPTER 4

Some miscellaneous inhibitors of ruminal methanogenesis in vitro

Abstract

Three inhibitors of methanogenesis that had not been previously tested in mixed ruminal fermentation were examined in vitro. The eukaryotic and archaeal DNA-polymerase inhibitor aphidicolin did not affect methanogenesis or most of the fermentation parameters. Interestingly, dimethylsulfoxide (DMSO), the carrier for aphidicolin delivery, caused a strong decrease in ammonia concentrations. Antiprotozoal properties of DMSO are suspected. The pterin, lumazine, decreased methanogenesis by about 50%. Fermentation was also inhibited. Surprisingly, molecular hydrogen accumulation was not observed and acetate molar proportion increased. The methyl-CoM analog 2-bromopropanesulfonate decreased fermentation and butyrate molar percentage and increased acetate molar percentage without affecting methane production. It is difficult to understand how a structural analog of a coenzyme that is unique to methanogens could have affected fermentation without altering methanogenesis.

Introduction

Methane formation in ruminants represents an energy loss and also makes some contribution to global warming. Hence, there is an interest in decreasing methane emissions by ruminant animals (Moss, 1993).

Aphidicolin is a specific inhibitor of DNA-polymerases types α, δ and ε in eukaryotes (Voet and Voet, 1995). It inhibited DNA replication in crude extracts of methanogens but not of *Escherichia coli*, and completely prevented the growth of several methanogens studied (Zabel et al., 1985). The effects of aphidicolin on ruminal fermentation have not been examined.

The pterin compound lumazine [2, 4-(1H, 3H)-pteridinedione] has been shown to inhibit the growth of several methanogens, but had little effect on other Archaea, bacteria or yeasts (Nagar-Anthal et al., 1996). Lumazine is a structural analog of some cofactors involved in methanogenesis, like methanopterin and deazaflavin F_{420} (White and Zhou, 1993). Its effects upon ruminal fermentation have not been studied.

2-Bromoethanesulfonate (BES) is a chemical analogue of coenzyme M (CoM), a cofactor involved in the last step of methanogenesis (McAllister et al., 1996). It inhibits methyl-CoM reductase as a competitive inhibitor with CoM (Smith, 1983), being a very specific and potent methanogenesis inhibitor. 3-Bromopropanesulfonate (BPS), however, was found to be a more potent inhibitor in the in vitro reaction, achieving 50% inhibition at a concentration 80-fold lower (Ellerman et al., 1989). To our knowledge, the effects of BPS on methanogenesis have not been studied with either pure or mixed microbial cultures.

The objectives of this work were to study the effects of aphidicolin, lumazine and BPS on ruminal fermentation of mixed ruminal cultures. It was hypothesized that these compounds would decrease CH_4 production.

Materials and Methods

Incubations

Ruminal fluid was withdrawn from two mature Holstein cows fed alfalfa hay, mixed and strained through four layers of cheesecloth. It was then blended for 15 s and strained through one layer of cheesecloth. One part of ruminal fluid was mixed with four parts of buffer (Goering and Van Soest, 1975), and 15 mL of the mixture anaerobically delivered into 25 mL Hungate tubes. All the tubes contained 0.2 g of finely ground alfalfa hay (11.4% CP DM basis) as substrate. Samples of the ruminal fluid/buffer mixture were taken and frozen to measure VFA initial concentrations. Tubes were sealed under an O_2-free CO_2 atmosphere and incubated in a shaking water bath at 39 °C for 24 h.

Treatments

The experimental treatments were: aphidicolin 0, 30, 60 and 150 μM, lumazine 0, 300, 600 and 1,200 μM, and BPS and BES (positive control) 0, 1, 10 and 50 μM. Final concentrations of aphidicolin were achieved by adding 46.5, 93 or 233 μL, respectively, of a 3.4 mg/μL solution of aphidicolin in dimethyl sulfoxide (DMSO; (Zabel et al., 1985) to Hungate tubes. The aphidicolin control received 233 μL of DMSO. Lumazine final concentrations were achieved by adding 0.8, 1.5 and 3.1 mg of lumazine to Hungate tubes. One hundred microliters of 360 ppm BPS and BES solutions were added to Hungate tubes to achieve a final concentration of 1 μM. Final BPS and BES concentrations of 10 and 50 μM were achieved by adding 0.10 and 0.50 mL, respectively, of 3600 ppm BPS and BES solutions to Hungate tubes. All tubes with 0.10 mL solutions

also received 0.40 mL of deionized water to equalize volumes. Control tubes for lumazine, BPS and BES treatments had 0.5 mL of deionized water added.

Analysis

At the end of the incubation, gas exceeding 1 atm was measured (Callaway and Martin, 1996) and the fermentation was stopped by adding 0.25 mL of a 10% phenol solution. A 0.1 mL gas sample was taken from each tube and analyzed for gas composition (CH_4, CO_2, and H_2) by gas chromatography (Gow Mac Gas Chromatograph series 750, Gow Mac Instruments Co., Bridgewater, NJ; carrier gas was N_2 at 820 KPa; 4' x 1/4" DC 200 column, s.s., operated at 150 °C; Gow Mac 069-50 Ruthenium Methanizer for CO_2 analysis; flame ionization detector for CH_4 and CO_2 analyses and RGD2 Reduction Gas Detector, Trace Analytical, Menlo Park, CA; for H_2 analysis; 3390 A Integrator, Hewlett Packard, Avondale, CA). A 5 mL aliquot was taken and centrifuged at 26,000 x *g* for 30 min. The pellet was discarded and the supernatant used for measuring pH (Digital Benchtop pH Meter, Cole-Parmer Instrument Company, Vernon Hills, IL) and VFA. VFA, lactate, formate and ethanol were analyzed by HPLC using a Waters 712 Wisp (Waters Associates Inc., Milford, MA), and separation was performed with a BioRad HPX 87H column (BioRad Laboratories, Hercules, CA). Detection was done by differential refractometry (Waters 410, Waters Associates Inc., Milford, MA). Ammonia was analyzed as described before (Chaney and Marbach, 1962).

Calculations

Apparently fermented organic matter (FOM) was estimated from the VFA stoichiometry (Demeyer and Van Nevel, 1979), but replacing caproate with isobutyrate: FOM (mg hexose) = (Ac/2 + Pro/2 + Isobut + But +Val + Isoval) x 162, where Ac is acetate, Pro is propionate, Isobut is isobutyrate, But is butyrate, Val is valerate and Isoval is isovalerate, all expressed in mmoles.

Statistical analysis

Five replicates per compound and concentration were used. Dependent variables were modeled as: observation = control + concentration + concentration^2 + concentration^3 + residual. Models with non-significant ($P > 0.05$) highest order coefficients were considered non-significant. A comparison between water and DMSO (aphidicolin) controls was done by the Scheffe test (Neter et al., 1996).

Results and Discussion

Aphidicolin

Aphidicolin addition did not affect methanogenesis, CO_2 release or H_2 accumulation (Table 4-1). Final pH, FOM, CH_4/FOM, total VFA concentration, acetate, propionate, and isovalerate production were similar. There were linear tendencies towards decreases in butyrate ($P = 0.07$) and isobutyrate ($P = 0.06$) production by maximums of 26 and 46% reductions, respectively. Valerate production peaked at 60 μM aphidicolin ($P < 0.01$; quadratic response). Aphidicolin at 60 μM was shown to completely inhibit the growth of five methanogens, and partially inhibited

Methanospirillum hungatei and *Methanosarcina barkeri* (Zabel et al., 1985). The growth of *Methanobacterium formicicum* was not affected at 60 μM, but it was completely inhibited at 210 μM. It is possible that the maximum concentration used in the present experiment, 150 μM, was insufficient to inhibit ruminal methanogens if they are intrinsically more resistant. The different sensitivities among methanogens could be caused by different cell envelopes (Zabel et al., 1985). These investigators found, however, that crude preparations of DNA-polymerases of *M. formicicum* and *Methanobacterium wolfei* were resistant to aphidicolin at 30 μM, whereas the activities of DNA-polymerases of *Methanococcus vannielii* were inhibited by 73%. *M. formicicum* DNA-polymerase activity was inhibited by about 20% at 150 μM of aphidicolin, which is the maximum concentration used in this experiment. Klimczak et al. (1986) found that DNA-polymerase of *Methanobacterium thermoautotrophicum* was insensitive to aphidicolin. Methanogens DNA-polymerases differ, therefore, in their sensitivity to aphidicolin. It is also possible that other ruminal microorganisms or substances present in ruminal fluid caused aphidicolin destruction and prevented inhibition of methanogens.

Table 4-1. Effects of aphidicolin on ruminal fermentation in vitro

	Aphidicolin (μM				Linear regression	Quadratic regression	Cubic regression	SEM[3]
	0	30	60	120	$P =$	$P =$[1]	$P =$[2]	
CH_4, μmol	52.1	35.9	54.9	58.1	0.48	0.65	0.30	28.1
H_2, μmol	0.06	0.12	0.07	0.15	0.16	0.76	0.24	0.08
CO_2, μmol	712	410	600	875	0.18	0.13	0.30	304
Total VFA, mM	70.1	69.2	68.9	66.1	0.20	0.83	0/89	4.94
Acetate, μmol	556	530	533	557	0.75	0.16	0.71	35.9
Propionate, μmol	209	199	190	189	0.34	0.61	0.91	32.4
Butyrate, μmol	97.3	106	104	71.9	0.07	0.12	0.99	24.4
Isobutyrate, μmol	37.2	44.6	33.4	20.2	0.06	0.37	0.43	16.7
Valerate, μmol	5.21	7.73	10.4	1.02	0.15	< 0.01	0.46	4.19
Isovalerate, μmol	8.31	10.4	21.2	7.3	0.98	0.19	0.41	15.4
FOM, %	42.2	41.4	40.9	39.2	0.18	0.94	0.92	3.52
CH_4/FOM, μmol/mg	0.68	0.47	0.73	0.81	0.35	0.61	0.31	0.38
Final pH	7.05	7.02	7.03	6.88	0.18	0.60	0.79	0.19

[1]Significance of the quadratic term.

[2]Significance of the cubic term.

[3]Standard error of the mean of the most significant model. If more than one model had a $P < 0.01$, the highest order model was used.

Surprisingly, NH_4^+ concentration was elevated by the addition of 30 μM aphidicolin, and to a lesser extent with 60 μM, but returned to control levels at 120 μM of aphidicolin (Table 4-2). Moreover, NH_4^+ concentration was substantially lower ($P < 0.01$; Scheffe analysis) in the DMSO control than in the water control (3.6 vs 17.5 mg/dL, respectively). It is then quite clear that this strong decrease in NH_4^+ was not caused by aphidicolin but by its vehicle, DMSO, as the DMSO controls received the same amount of DMSO as the 120 μM treatment.

Although part of the drop in NH_4^+ concentrations could be explained by a trend ($P = 0.18$) towards less fermentation, the decline was too pronounced to be accounted for solely by this. It is possible that DMSO had antiprotozoal properties, as defaunation decreases ruminal NH_4^+ concentration (Williams and Coleman, 1997). Alternatively, it might have inhibited proteolytic bacteria. A reduction in protein degradation and intraruminal N recylcling can be beneficial as it can result in a greater flow of amino acids to the small intestine (Nagaraja et al., 1997). Further research is needed into the mechanisms by which DMSO lowered ruminal NH_4^+ concentrations.

Table 4-2. Effects of aphidicolin and DMSO on ammonia levels and FOM

Treatment	NH_4^+ (mg/ 100 mL)	FOM (%)
Water control (no DMSO)	17.5	43.5
Aphidicolin control (233 μL DMSO)	3.36	42.2
Aphidicolin 30 μM (46.5 μL DMSO)	9.45	41.4
Aphidicolin 60 μM (93 μL DMSO)	5.92	40.9
Aphidicolin 120 μM (233 μL DMSO)	2.97	39.2
SEM	1.05[1, 2]	3.51[1, 3]

[1]Regressions do not include the water controls.
[2]Cubic regression ($P < 0.01$).
[3]Linear regression ($P = 0.18$).

Lumazine

Addition of lumazine at 600 and 1,200 μM decreased ($P < 0.01$) CH_4 formation by 50% of the control (Figure 4-1). Dihydrogen accumulation was not affected. Carbon dioxide release decreased in a quadratic ($P < 0.05$) fashion. There was little benefit of increasing lumazine beyond 600 μM. Lumazine at 450 or 600 μM strongly inhibited growth of the four methanogens studied, which included non-ruminal strains of *Methanobacterium formicicum* and *Methanosarcina barkeri* (Nagar-Anthal et al., 1996). Apparently fermented OM was decreased ($P < 0.01$; quadratic response) by 12, 17 and 17% by lumazine at 300, 600 and 1,200 μM, respectively (Table 4-3). A decline in fermentation with methanogenesis inhibitors has previously been observed (Prins, 1965; Chalupa et al., 1980). and attributed to an interference with the interspecies H transfer, which is necessary for the reoxidation of cofactors (Nagaraja et al., 1997). The final pH

which is necessary for the reoxidation of cofactors (Nagaraja et al., 1997). The final pH and NH_4^+ concentration were not affected by lumazine addition. Methane produced per milligram of FOM tended ($P = 0.05$) to decrease with lumazine addition.

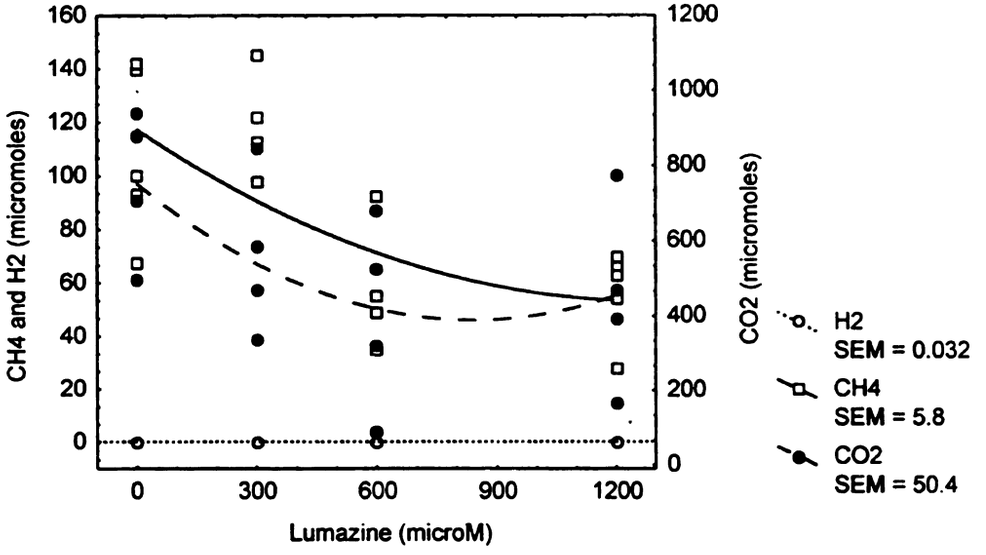

Figure 4-1 Effects of lumazine on gases production in ruminal fermentation

Total VFA concentration was decreased ($P < 0.01$; quadratic response; Table 4-3) by lumazine by a maximum of 12% at 600 μM. Acetate and valerate productions were not affected. Propionate production tended ($P = 0.07$) to linearly decline by a maximum of 11%. Butyrate production fell ($P < 0.01$; quadratic response) by 60%. Isobutyrate production dropped ($P < 0.01$; quadratic response) by more than 10-fold at 600 μM lumazine, although it recovered to control levels at 1,200 μM. Isovalerate production was linearly decreased ($P = 0.02$) by a maximum of 29%.

Table 4-3. Effects of the addition of lumazine on in vitro ruminal fermentation

	Initial concentration, μM				Linear regression $P =$	Quadratic regression $P =$[1]	Cubic regression $P =$[2]	SEM[3]
	0	300	600	1,200				
Total VFA, mM	71.1	65.9	62.9	65.2	0.02	< 0.01	0.88	2.59
Acetate, μmol	527	549	538	540	0.67	0.45	0.35	27.7
Propionate, μmol	197	193	177	176	0.07	0.53	0.48	9.17
Butyrate, μmol	134	66.9	53.0	53.5	< 0.01	< 0.01	0.07	15.4
Isobutyrate, μmol	45.0	10.3	4.21	42.2	0.75	< 0.01	0.51	11.0
Valerate, μmol	13.4	12.8	12.5	12.5	0.25	0.50	> 0.99	1.22
Isovalerate, μmol	11.6	9.78	8.54	8.27	0.02	0.20	0.96	2.07
Acetate to propionate	2.74	2.84	3.05	3.07	0.03	0.37	0.53	0.24
FOM, %	43.5	38.4	36.1	36.1	< 0.01	< 0.01	0.70	1.57
CH_4/FOM, μmol/mg	1.36	1.36	0.81	0.84	0.05	0.95	0.28	0.063
Final pH	6.92	6.96	7.06	6.99	0.28	0.14	0.34	0.10
NH_4^+, mg/dL	17.5	17.4	17.6	16.8	0.24	0.46	0.62	9.91

[1]Significance of the quadratic term.
[2]Significance of the cubic term.
[3]Standard error of the mean of the most significant model. If more than one model had a $P < 0.01$, the highest order model was used.

It is unusual that the acetate to propionate ratio increased ($P = 0.03$; Table 4-3), as the inhibition of methanogenesis consistently shifts the VFA profile from acetate to propionate (Nagaraja et al., 1997). The inhibition of methanogenesis was accompanied by a decrease in CO_2 release and H_2 accumulation did not increase. It is possible that some of the electrons spared from methanogenesis were redirected towards acetate synthesis from CO_2 and H_2, thus compensating for the shift of fermentation from acetate

to propionate. Acetogens synthesize acetate using the same precursors as methanogens, but they do not compete well with methanogens under normal ruminal conditions due to their higher thermodynamic threshold for H_2 (Nagaraja et al., 1997). Nevertheless, it was shown that when methanogenesis was inhibited, reductive acetogenesis increased when the acetogen *Peptostreptococcus productus* was added to the fermentation medium (Nollet et al., 1997). It is tempting to think that lumazine could somehow have stimulated ruminal acetogens.

BPS

BPS did not affect CH_4 production (Table 4-4), whilst BES decreased ($P < 0.01$) it by 50 and 48% at 10 and 50 μM, respectively (Table 4-5). BES effects on methanogenesis were in agreement with previous observations (Martin and Macy, 1985; Nollet et al., 1997). BPS was, however, expected to be a more potent inhibitor according to previous observations with a pure preparation of methyl-CoM reductase from *Methanobacterium thermoautotrophicum* (Ellerman et al., 1989). BPS greater effectiveness as an inhibitor of methyl-CoM reductase is due to a more similar structure to methyl-CoM as compared to BES (Ellerman et al., 1989). This should not change even if methyl-CoM reductase of ruminal methanogens is different from the one of *M. thermoautotrophicum*. There is, therefore, no clear explanation on the lack of effectiveness of BPS in decreasing ruminal methanogenesis.

Table 4-4. Effects of the addition of BPS on in vitro ruminal fermentation

	Initial concentration, μM				Linear regression $P =$	Quadratic regression $P =$[1]	Cubic regression $P =$[2]	SEM[3]
	0	1	10	50				
CH_4, μmol	109	75.0	89.6	103	0.21	0.61	0.25	30.5
H_2, μmol	0.29	0.24	0.33	0.33	0.59	0.56	0.66	0.19
CO_2, μmol	746	560	694	682	0.70	0.56	0.78	294
Total VFA, mM	71.1	63.8	65.7	64.4	0.15	0.34	< 0.01	2.52
Acetate, μmol	527	539	538	534	0.97	0.56	0.43	21.2
Propionate, μmol	197	174	182	177	0.51	0.73	0.14	23.5
Butyrate, μmol	134	65.9	78.5	72.8	0.13	0.16	< 0.01	30.0
Isobutyrate, μmol	45.0	6.75	18.2	11.5	0.15	0.37	< 0.01	10.4
Valerate, μmol	13.4	13.6	13.1	13.1	0.47	0.58	0.67	0.97
Isovalerate, μmol	11.6	9.51	9.02	10.3	0.99	0.24	0.27	2.67
FOM, %	43.5	37.2	38.6	37.7	0.11	0.24	< 0.01	1.57
CH_4/FOM, μmol/mg	1.36	1.10	1.26	1.49	0.29	0.85	0.37	
Final pH	6.92	7.01	6.86	6.95	0.97	0.23	0.16	0.11
NH_4^+, mg/dL	17.5	18.2	17.8	17.9	0.83	0.97	0.27	0.88

[1]Significance of the quadratic term.
[2]Significance of the cubic term.
[3]Standard error of the mean of the most significant model. If more than one model had a $P < 0.01$, the highest order model was used.

BPS did not affect H_2 or CO_2 release, acetate, propionate, valerate, or isovalerate production, CH_4 production per milligram of FOM, final pH, or NH_4^+ concentration. BPS decreased ($P < 0.01$) FOM from 43.5 to 37.7%. Total VFA concentration, butyrate and isobutyrate production were decreased ($P < 0.01$). It is difficult to understand how BPS could have altered decreased butyrate and isobutyrate production. A major part of

butyrate production is carried out be *Butyribivrio fibrisolvens* (Stewart et al., 1997) and protozoa (Huhtanen, 1992; Jaakkola and Huhtanen, 1993). Isobutyrate is a product of valine fermentation (Voet and Voet, 1995). As CoM is a cofactor unique to methanogens (Balch and Wolfe, 1979), methyl-CoM analogs, like BPS, should not affect other ruminal microorganisms.

Table 4-5. Effects of the addition of BES on in vitro ruminal fermentation

	Initial concentration, μM				Linear regression $P =$	Quadratic regression $P =$[1]	Cubic regression $P =$[2]	SEM[3]
	0	1	10	50				
CH_4, μmol	109	119	54.0	56.4	0.01	< 0.01	< 0.01	21.7
H_2, μmol	0.29	0.35	0.15	0.26	< 0.01	< 0.01	0.63	1.95
CO_2, μmol	746	562	403	456	0.13	0.22	0.22	255
Total VFA, mM	71.1	65.9	62.9	65.2	0.27	0.16	< 0.01	2.29
Acetate, μmol	527	549	538	540	0.01	< 0.01	0.88	18.2
Propionate, μmol	197	193	177	176	< 0.01	0.02	0.81	23.9
Butyrate, μmol	134	66.9	53.0	53.5	0.14	0.16	< 0.01	15.0
Isobutyrate, μmol	45.0	10.3	4.21	42.2	0.10	0.06	< 0.01	8.85
Valerate, μmol	13.4	12.8	12.5	12.5	< 0.01	< 0.01	0.24	1.38
Isovalerate, μmol	11.6	9.78	8.54	8.27	0.43	0.81	0.30	2.58
FOM, %	43.5	38.5	36.1	36.1	0.20	0.14	< 0.01	1.42
CH_4/FOM, μmol/mg	1.36	1.36	0.81	0.84	0.01	< 0.01	0.02	0.29
Final pH	6.92	6.96	7.06	6.99	0.67	0.27	0.94	0.14
NH_4^+, mg/dL	17.5	17.4	17.6	16.8	0.12	0.37	0.79	12.7

[1]Significance of the quadratic term.
[2]Significance of the cubic term.
[3]Standard error of the mean of the most significant model. If more than one model had a $P < 0.01$, the highest order model was used.

Conclusions

Aphidicolin and 2-bromopropanesulfonate did not decrease ruminal methanogenesis in vitro. Aphidicolin's vehicle DMSO, lowered NH_4^+ concentration by more than 80%, and could act as an antiprotozoal agent. More research is needed to establish how DMSO lowered NH_4^+ concentration. Lumazine decreased CH_4 production by about 50%, although fermentation was decreased. Unexpectedly, the acetate to propionate ratio increased. The effects of lumazine on pure cultures of ruminal methanogens and acetogens need to be evaluated.

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CHAPTER 5

Effects of two lipids on in vitro ruminal methane production

Abstract

As CH_4 emissions by ruminants are a loss of energy and also contribute to global warming, there is an interest in decreasing rumen methanogenesis. Fats and oils usually decrease CH_4 production both in vitro and in vivo, although they can inhibit fermentation. The effects of olive oil and a hexadecatrienoic acid (cis-$C_{16:6,9,12}$) on mixed ruminal cultures were studied in 24 h-batch fermentation. The hexadecatrienoic acid was extracted from the Hawaiian algae *Chaetoceros*. Initial concentrations of both oils were 0.5, 1 and 2 mL/L. The hexadecatrienoic acid linearly decreased CH_4 production by 97 %, while olive oil did not affect it. The hexadecatrienoic acid also increased H_2 accumulation. Release of CO_2 was linearly decreased by the hexadecatrienoic acid, while olive oil increased it linearly. None of the oils had an effect on final pH. Apparently fermented OM, as estimated through the VFA stoichiometry, was linearly decreased by the hexadecatrienoic acid by 42%, while olive oil did not affect it. The hexadecatrienoic acid decreased acetate and butyrate production, while propionate production peaked at 1 mL/L. Olive oil tended to decrease acetate production and increased propionate and butyrate. The hexadecatrienoic acid linearly decreased NH_4^+ concentration. The hexadecatrienoic acid was a strong inhibitor of methanogenesis, but it decreased fermentation and increased H_2 accumulation. Olive oil could be used to increase dietary energy without negatively affecting fermentation.

Introduction

Methane emissions by ruminants represent a loss of energy and also contribute to global warming (Moss, 1993). Therefore, there is an economic and environmental interest in decreasing CH_4 formation in the rumen. Unsaturated fatty acids could compete with methanogenesis for reducing equivalents during biohydrogenation in the rumen (Czerkawski et al., 1966a, b).

Fats and oils have a variety of effects on ruminal fermentation and microbial activities. There is a consistent decrease in ciliate protozoal numbers (Nagaraja et al., 1997; Machmüller and Kreuzer, 1998; Machmüller et al., 1998). Methanogenesis is decreased in vitro and in vivo, and the VFA profile is generally shifted from acetate and butyrate towards propionate. However, the inhibition of CH_4 production cannot be fully accounted for by biohydrogenation competing for metabolic H or by the elimination of methanogens associated with ciliate protozoa (Nagaraja et al., 1997). Direct toxicity on methanogens has been shown in mixed (Dong et al., 1997) and pure culture studies (Prins et al., 1972). The reasons for oil toxicity on methanogens and Gram positive bacteria are poorly understood, but they may involve alterations in cell membrane permeability that affect nutrient uptake and regulation of intracellular pH (Nagaraja et al., 1997).

A problem associated to the use of fats and oils is an inhibition of ruminal fermentation of non-lipid energy sources, especially fiber (Nagaraja et al., 1997), although some products like canola oil or cod liver oil have not affected NDF disappearance (Dong et al., 1997). The objective of the present study was to examine the effects of a hexadecatrienoic acid ($C_{16:6,9,12}$) isolated from the Hawaiian marine algae

Chaetoceros, and olive oil predominant fatty acids, on fermentation by mixed ruminal cultures. It was hypothesized that the oils would decrease CH_4 production.

Materials and Methods

Oils and concentrations

An unsaturated hexadecatrienoic fatty acid (cis-$C_{16:6,9,12}$), isolated from the Hawaiian marine algae *Chaetoceros* (courtesy of J. –K. Wang, Department of Biosystems and Engineering, University of Hawaii), and food grade olive oil (Pompeian Inc., Baltimore, MD) were examined as potential inhibitors of ruminal methanogenesis in vitro. Initial concentrations of both oils were 0.5, 1, and 2 mL/L. Densities of the hexadecatrienoic fatty acid and olive oil were 0.90 and 0.91 g/mL, respectively. Initial concentrations were achieved by adding 25, 50, and 100 μL of each oil to 125 mL-Wheaton bottles.

Ruminal fluid collection and incubation

Ruminal fluid was withdrawn prior to the morning feeding from two mature Holstein cows fed alfalfa hay. It was mixed together, and strained through two layers of cheesecloth. It was then blended for 15 s, and again strained through two layers of cheesecloth. One volume part of ruminal fluid was mixed with four volume parts of a bicarbonate and phosphate buffer (Goering and Van Soest, 1975), and 50 mL of the mixture anaerobically delivered into 125 mL-Wheaton bottles. All the bottles contained 250 mg of ground (0.2 mm screen mesh) alfalfa hay (1.8% N and 3.5% ash on a DM basis) as substrate. Three samples of the ruminal fluid and buffer mixture were frozen for

subsequent determination of VFA initial concentrations. Bottles were sealed under an O_2-free CO_2 atmosphere, and incubated in a shaking water bath at 39 °C for 24 h. At the end of the incubation, bottles were allowed to cool to room temperature, and total gas production volume was measured (Callaway and Martin, 1996). Fermentation was then stopped by adding 1 mL of a 10% phenol solution.

Analytical procedures

Methane and CO_2 were analyzed (Callaway and Martin, 1996) using a Gow Mac series 750 flame ionization detector gas chromatograph (Gow Mac Instruments Co., Bridgewater, NJ) equipped with a 4' x 1/4" DC 200 stainless steel column (150 °C, carrier gas was N_2 at 820 kPa). A RGD2 Reduction Gas Detector (Trace Analytical, Menlo Park, CA), equipped with the same column, was used for H_2 analysis. The volume of gas produced was expressed as μmoles at 25 °C and 1 atm. A 5 mL-aliquot was centrifuged (26,000 x *g*, 4 °C, 30 min) and the pH was measured in the supernatant (Digital Benchtop pH Meter, Cole-Parmer Instrument Company, Vernon Hills, IL). Volatile fatty acids, lactate, formate, ethanol, and the chemical additives were quantified by differential refractometry with a Waters 712 Wisp HPLC (Waters Associates Inc., Milford, MA) equipped with a BioRad HPX 87H column (BioRad Laboratories, Hercules, CA). Separation was done by ion moderated partition. Solvent was 0.005 M H_2SO_4 at 0.6 mL/min. Column temperature was 65 °C. Sample injection volume was 15 μL. Ammonia was analyzed as reported before (Chaney and Marbach, 1962).

Calculations

Apparently fermented substrate OM (FOM) was estimated from the VFA stoichiometry (Demeyer and Van Nevel, 1979), but using isobutyrate instead of caproate: apparently fermented substrate (%) = (Acetate/2 + Propionate/2 + Isobutyrate + Butyrate + Valerate + Isovalerate) x 162 / substrate OM (mg), with all VFA expressed in millimoles produced. It was assumed the oils were not fermented to VFA.

Statistical analysis

Four replications per oil and initial concentration were used. Responses were modeled as: observation = control + concentration + concentration^2 + concentration^3 + residual. Models with non-significant ($P > 0.05$) highest order coefficients were considered non-significant.

Results and Discussion

The hexadecatrienoic acid decreased ($P < 0.01$) CH_4 production by 97% (Table 5-1). Possibly, the highly unsaturated hexadecatrienoic acid was toxic to methanogens (Prins et al., 1972). In contrast, olive oil did not affect CH_4 production (Table 5-1). Lack of effect of other oils on ruminal methanogenesis has also been reported for protected fat and crushed canola seed (Machmüller et al., 1998). Oleic acid is the main component of both olive oil (Kirisakis and Christie, 2000) and canola oil (Dong et al., 1997). However, a product containing 74.6% oleic acid at a concentration of 0.5 g/L (equivalent to 0.37 g/L of olive oil) inhibited the growth of *Methanobrevibacter ruminantium*, regarded as the main ruminal methanogen (Sharp et al., 1998), by 82% (Henderson, 1973). In the

current experiment, 2 mL/L of olive oil would be expected to contain at least 1.1 g/L of oleic acid (Kirisakis and Christie, 2000). Given the high percentage of oleic acid in olive oil, it is difficult to explain the lack of effect on CH_4 production. Sensitivity of bacteria to fatty acids is related to cell wall structure (Galbraith et al., 1971; Galbraith and Miller, 1973). The cell envelope of *Methanobrevibacter* differs from other ruminal methanogens in the presence of a pseudomurein layer instead of an S-layer (Sprott and Beveridge, 1993). It is possible that the presence of an S-layer confers other ruminal methanogens some protection against fatty acid toxicity. Toxicity of oleic acid on pure cultures of ruminal methanogens other than *Methanobrevibacter* needs to be investigated.

A 5-fold increase ($P < 0.01$) in H_2 accumulation was observed at the maximum hexadecatrienoic acid concentration (Table 5-1). An increase in H_2 accumulation is often a consequence of the inhibition of methanogenesis (Martin and Macy, 1985). Although there is no stoichiometrical relationship, several experiments have shown greater H_2 accumulation with stronger methanogenesis inhibition (Dong et al., 1997; Machmüller and Kreuzer, 1998). Similarly, in the present study, H_2 accumulation increased about by 2-fold with 0.5 and 1 mL/L hexadecatrienoic acid, with a 25 and 53% decrease in CH_4 production, respectively, and by 5-fold with 97% inhibition of methanogenesis at 2 mL/L.

Table 5-1. Effects of a hexadecatrienoic oil and olive oil on ruminal fermentation in vitro

	Initial concentration, mL/L				Linear effect $P =$	Quadratic effect $P =$[1]	Cubic effect $P =$[2]	SEM[3]
	0	0.5	1	2				
Hexadecatrienoic acid								
CH_4, µmol	336	253	158	10.4	< 0.01	0.80[1]	0.85[2]	76.2[3]
CO_2, µmol	1777	1608	1210	918	< 0.01	0.49	0.29	201
H_2, µmol	11.2	21.3	21.6	57.6	< 0.01	0.45	0.60	22.1
Total VFA, mM	43.1	40.5	37.4	29.3	< 0.01	0.51	0.96	3.11
Total VFA production, µmol	1581	1447	1292	877	< 0.01	0.52	0.96	159
Acetate, µmol	1093	953	799	478	< 0.01	0.86	0.97	141
Propionate, µmol	336	357	373	321	0.42	0.01	0.64	31.9
Butyrate, µmol	111	102	87	50	< 0.01	0.55	0.94	20.4
Isobutyrate, µmol	-1.67	-3.14	-2.27	-3.81	0.02	> 0.99	< 0.05	0.97
Valerate, µmol	19.9	21.4	23.2	22.9	0.34	0.57	0.86	4.55
Isovalerate, µmol	21.9	14.8	10.3	5.0	< 0.01	0.33	0.89	5.90
Apparently fermented substrate, %	60.8	55.5	49.5	35.1	< 0.01	0.69	> 0.99	6.31
CH_4/FOM, µmol/mg	2.40	1.90	1.26	0.17	< 0.01	0.99	0.84	0.55
Final pH	6.97	6.95	6.95	6.99	0.67	0.42	0.97	0.069
NH_4^+, mg/dL	18.9	17.9	17.9	14.9	< 0.01	0.79	0.59	1.41
Olive oil								
CH_4, µmol	336	338	341	340	0.51	0.58	0.86	9.01
CO_2, µmol	1777	1934	1810	2072	0.02	0.47	0.11	146
H_2, µmol	11.2	10.1	8.6	9.5	0.71	0.69	0.91	6.58
Total VFA, mM	43.1	42.5	43.4	43.5	0.29	0.73	0.16	0.77
Total VFA production, µmol	1581	1554	1595	1597	0.38	0.75	0.21	41.0
Acetate, µmol	1093	1090	1068	1047	0.05	0.94	0.68	31.2
Propionate, µmol	336	343	347	365	< 0.01	0.71	0.73	10.6
Butyrate, µmol	111	96.1	136	153	0.02	0.69	0.10	25.3
Isobutyrate, µmol	-1.67	-3.79	1.21	2.42	0.24	0.87	0.37	5.82
Valerate, µmol	19.9	10.1	14.2	9.40	0.22	0.58	0.27	8.74
Isovalerate, µmol	21.9	14.7	28.8	22.3	0.59	0.74	0.07	9.51
Apparently fermented substrate, %	60.8	58.6	62.3	62.7	0.14	0.72	0.06	2.27
CH_4/FOM, µmol/mg	2.40	1.88	2.38	2.36	0.80	0.63	0.27	0.65
Final pH	6.97	6.96	6.89	6.96	0.76	0.29	0.49	0.094
NH_4^+, mg/dL	18.9	19.6	18.1	19.2	0.94	0.67	0.13	0.95

[1]Significance of the quadratic term.

[2]Significance of the cubic term.

[3]Standard error of the mean of the most significant model. If more than one model has a $P < 0.01$, the highest order model was used.

There was a 16-fold increase in formate accumulation at 2 mL/L hexadecatrienoic acid, although no formate accumulated at the lower concentrations ($P = 0.04$; quadratic response; data not shown). Formate is used as a CH_4 precursor in the rumen (Asanuma et al., 1998). The hexadecatrienoic acid also increased ($P < 0.05$; cubic response; data not shown) ethanol production. Interestingly, there was a 4.6- and 9-fold increase ($P < 0.01$; data not shown) in formate accumulation at 1 and 2 mL/L olive oil, respectively. The increase in this unusual H sink is difficult to understand, as olive oil did not inhibit CH_4 production. As there was a cubic tendency ($P = 0.06$; Table 5-1) for olive oil at 1 and 2 mL/L to increase OM apparent fermentability, it is possible that higher rates of fermentation than formate utilization by methanogens (Asanuma et al., 1998) or succinate producers caused some increase in formate accumulation.

There was a linear decrease ($P < 0.01$) in CO_2 release with the hexadecatrienoic acid (Table 5-1). In previous results (Dong et al., 1997), supplementation with coconut oil, but not with canola oil, caused a decrease in CO_2 release. Cod liver oil caused a slight decrease in CO_2 release when supplementing grass hay, but not with concentrate (Dong et al., 1997). In contrast, in the present study the release of CO_2 was increased ($P = 0.02$) by olive oil by 17% at 2 mL/L. This is difficult to reconcile with the changes it caused in VFA production. A decrease ($P = 0.05$) in acetate of 46 μmol, an increase in propionate of 29 μmol ($P < 0.01$), and an increase in butyrate of 42 μmol ($P = 0.02$), should all result in a net release of 47 μmol CO_2 (CO_2 = acetate/2 + propionate/4 + 1.5 x butyrate; (Blümmel et al., 1997). This is clearly insufficient to explain the net increase observed in CO_2 release of approximately 300 μmol.

The hexadecatrienoic acid decreased ($P < 0.01$) acetate and butyrate production by more than 50%. Propionate production peaked at 1 mL/L hexadecatrienoic acid and then decreased ($P = 0.01$; quadratic response). Olive oil increased ($P < 0.01$) propionate production by 9%. This is important, as propionate is the main glucose precursor in ruminants (Nagaraja et al., 1997). Both oils decreased ($P < 0.01$; data not shown) the acetate to propionate ratio. Fats and oils generally, but not always, shift the VFA profile from acetate to propionate (Nagaraja et al., 1997). The hexadecatrienoic acid tended ($P = 0.07$; data not shown) to decrease butyrate molar percentage. Butyrate molar percentage is generally decreased by fats and oils as protozoal numbers and activities are decreased (Nagaraja et al., 1997). On the contrary, olive oil increased ($P = 0.02$; data not shown) butyrate molar percentage. In agreement, canola oil, which is also rich in oleic acid (Dong et al., 1997), did not affect butyrate molar percentage or protozoal numbers in continuous culture (Machmüller et al., 1998). Other results have shown a decrease in butyrate molar percentage when supplementing a concentrate diet, but not a roughage diet, with canola (Dong et al., 1997).

There was a net disappearance of isobutyrate in all treatments. The reasons for this are not clear, as isobutyrate initial concentration was not unusually high as compared to similar experiments (data not shown). The hexadecatrienoic acid caused a 4-fold decrease ($P < 0.01$) in isovalerate production. Isovalerate can be produced by the catabolism of leucine (Voet and Voet, 1995). The hexadecatrienoic acid did inhibit deamination, as reflected by the linear decrease ($P < 0.01$) in NH_4^+ concentration, which is a consistent result from oil supplementation (Nagaraja et al., 1997). Olive oil did not

seem to affect deamination, as reflected by the lack of change in NH_4^+ and isoacids concentrations.

The hexadecatrienoic acid caused a linear decrease ($P < 0.01$) in the alfalfa hay substrate apparent fermentation and CH_4 produced per milligram of FOM. In contrast, supplementation with olive oil did not affect the substrate fermentation or CH_4 produced per milligram of FOM. Digestibility of roughage diets has been shown to be decreased by coconut oil (Dong et al., 1997; Machmüller and Kreuzer, 1998; Machmüller et al., 1998) but not by protected fat, crushed canola seed, crushed linseed (Machmüller et al., 1998), canola or cod liver oil (Dong et al., 1997). Likely, the high degree of unsaturation of the hexadecatrienoic acid was toxic for *Ruminococcus flavefaciens* and *R. albus*, which are important cellulolytic bacteria with a Gram-positive-type cell wall (Nagaraja et al., 1997).

Oleic acid, which is the most abundant fatty acid in olive oil (Kirisakis and Christie, 2000), did not affect ruminal Gram negative bacteria (Henderson, 1973; Maczulak et al., 1981), but was strongly inhibitory for *Butyrivibrio* B 835 and *Ruminococcus* 4263/1. A product containing 74.6% oleic acid at a concentration of 0.1 g/L inhibited the growth of these species by more than 80% (Henderson, 1973). Interestingly, oleic acid at lower concentrations (0.01 g/L or less) was actually stimulatory for *Butyrivibrio* B 835 (Henderson, 1973; Maczulak et al., 1981), although it inhibited other strains of *B. fibrisolvens* (Maczulak et al., 1981). One strain of *Ruminococcus albus*, and two strains of *R. flavefaciens* were strongly inhibited by oleic acid, even at a concentration as low as 0.005 g/L (Maczulak et al., 1981). Hence, it is difficult to explain why olive oil at 2 mL/L (which would represent at least 1 mL/L, or

0.891 g/L, of oleic acid) did not inhibit fermentation or CH_4 production. It is possible that Gram negative cellulolytic species, like *Fibrobacter succinogenes* (Maczulak et al., 1981), could have colonized fiber surfaces left by the inhibited Ruminococci. Also, as large strain differences can occur (Henderson, 1973), it is possible that different strains of the same species were present during the current experiment incubations and were less affected.

Alternatively, cholesterol and ergocalciferol at 0.45 mM have caused some reversal of the inhibition caused by Na lauryl sulfate, lauric, and linoleic acids on non-ruminal microorganisms (Galbraith et al., 1971). The presence of steroids in olive oil (Kirisakis and Christie, 2000) may have relieved the inhibition caused by long-chain fatty acids. However, the concentrations of steroids in the present study incubations should have been much lower than levels reported to reverse the fatty acid inhibition.

In agreement with the present results, the effects of oil supplementation on ruminal pH are generally minor (Dong et al., 1997; Nagaraja et al., 1997; Machmüller et al., 1998).

Conclusions

The hexadecatrienoic acid extracted from the Hawaiian algae *Chaetoceros* was a very strong inhibitor of ruminal methanogenesis in vitro. Before it could be used as an inhibitor of methanogenesis in vivo, it would be necessary to overcome its negative effects on fermentation, as well as the increase it caused in H_2 and formate accumulation, and ethanol production. Perhaps the addition of alternative electron acceptors, or acetogens (Nollet et al., 1997), could rechannel electrons away from H_2, formate and

ethanol, and also improve fermentation. Although olive oil did not inhibit CH_4 production, it could be used to increase the energy content of ruminant diets and the supply of propionate, the main glucose precursor, without depressing ruminal fermentation.

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CHAPTER 6

Effects of combinations of inhibitors of methanogenesis with crotonic acid or 3-butenoic acid on in vitro ruminal fermentation and methane production

Abstract

It was hypothesized that the reduction of crotonate or 3-butenoate to butyrate would utilize reducing equivalents not incorporated into CH_4 formation when the latter is inhibited by lumazine, propynoic acid, or ethyl 2-butynoate. This would avoid the accumulation of unusual reduced products without a nutritional value (H_2, formate, and ethanol). In six experiments, three inhibitors of CH_4 formation (lumazine, propynoic acid, and ethyl 2-butynoate), each at three different initial concentrations, were combined with either crotonic acid or 3-butenoic acid, each at two different initial concentrations. A 4:1 buffer and ruminal fluid mixture was incubated in Wheaton bottles for 24 h, using ground alfalfa hay as substrate. The inhibition of methanogenesis caused by lumazine was smaller than previously observed, and there was no accumulation of reduced end products without a nutritional value. Propynoic acid at its maximum initial concentration decreased CH_4 production by more than two thirds. Crotonic acid and 3-butenoic acid were ineffective in avoiding the accumulation of H_2 and formate or decreasing ethanol production. Ethyl 2-butynoate suppressed methanogenesis by more than 90%. Crotonic acid caused some decrease in H_2 accumulation and ethanol production, while 3-butenoic acid was ineffective. Crotonic acid and 3-butenoic acid were ineffective in avoiding the formation of unusual reduced products partly because they were also metabolized to acetate, thereby releasing, rather than incorporating, reducing equivalents. Incomplete

disappearance of crotonic acid could also explain the lack of effectiveness of this additive.

Introduction

Ruminal methanogenesis represents a substantial loss of energy to the animal and is also a major source of greenhouse gas emissions from agriculture (Newbold et al., 2001). However, methanogenesis is the main means of disposal of metabolic H in ruminal fermentation (Newbold et al., 2001) and its inhibition can decrease fermentation as the interspecies transfer of H_2 is disrupted and reduced cofactors do not get reoxidized (Nagaraja et al., 1997). Propynoic acid and ethyl 2-butynoate decreased methanogenesis in vitro by over 70%; however, total OM (substrate plus additive) apparent fermentability was decreased by both compounds. Lumazine also decreased methanogenesis by about 50%, but inhibited fermentation (Ungerfeld et al., 2000). Ethyl 2-butynoate also inhibited the fermentation of the alfalfa hay substrate. These compounds also caused the formation of unusual products of fementation, like H_2, ethanol, and formate, without a nutritional value to the host animal.

β-Hydroxybutyrate, crotonic acid, and 3-butenoic acid, had little effects on methanogenesis, but seemed to stimulate fermentation of the alfalfa hay substrate. It is possible that these organic acids benefited fermentation by acting as alternative electron sinks. The inhibition of methanogenesis might have caused the formation of unusual reduced products by disrupting the interspecies H transfer. It was hypothesized that the combination of inhibitors of methanogenesis and compounds that stimulated fermentation would lift the constraints caused by the former. The objective of this series of six

experiments was to examine the effects of combinations of lumazine, propynoic acid, and ethyl 2-butynoate, with crotonic acid or 3-butenoic acid, on fermentation of mixed ruminal cultures.

Material and Methods

Experiment 1

The experimental treatments of the 3 x 2 factorial arrangement were: 1) Lumazine (Sigma L 0380 for all experiments) 0 mM, crotonic acid (Sigma C4630 for all experiments) 0 mM (double control); 2) Lumazine 0 mM, crotonic acid 8 mM; 3) Lumazine 0.3 mM, crotonic acid 0 mM; 4) Lumazine 0.3 mM, crotonic acid 8 mM; 5) Lumazine 0.6 mM, crotonic acid 0 mM; 6) Lumazine 0.6 mM, crotonic acid 8 mM. Lumazine was added as a solid, while crotonic acid was added as a 1 mL-solution. Crotonic acid controls received 1-mL of deionized water.

Ruminal fluid was withdrawn prior to the morning feeding from two mature, non-lactating Holstein cows fed alfalfa hay. It was mixed, and strained through two layers of cheesecloth. It was then blended for 15 s and again strained through two layers of cheesecloth. One volume part of ruminal fluid was mixed with four volume parts of buffer (Goering and Van Soest, 1975) and 80 mL of the mixture anaerobically delivered into 160-mL Wheaton bottles. All the bottles contained 500 mg of ground (0.2 mm mesh screen) alfalfa hay (1.8% N, DM base) as substrate. Lumazine was added to the corresponding bottles as a solid, while crotonic acid was added as a solution (1 mL/bottle). Crotonic acid controls received 1 mL of deionized water. Three samples of the ruminal fluid and buffer mixture were frozen for subsequent determination of VFA

initial concentrations. Bottles were sealed under an O_2-free CO_2 atmosphere and incubated in a shaking water bath at 39 °C for 24 h. At the end of the incubation, bottles were allowed to cool to room temperature and total gas production volume was measured (Callaway and Martin, 1996). Fermentation was then stopped by adding 1 mL of a 10% phenol solution.

Methane and CO_2 were analyzed (Callaway and Martin, 1996), using a Gow Mac series 750 flame ionization detector gas chromatograph (Gow Mac Instruments Co., Bridgewater, NJ) equipped with a 4' x 1/4" DC 200 column (150 °C, carrier gas was N_2 at 820 Kpa). A RGD2 Reduction Gas Detector (Trace Analytical, Menlo Park, CA), equipped with the same type of column, was used for H_2 analysis. Gas production was expressed as micromoles at 25 °C and 1 atm. A 5-mL aliquot was centrifuged (26,000 x *g*, 4 °C, 30 min) and pH measured in the supernatant (Digital Benchtop pH Meter, Cole-Parmer Instrument Company, Vernon Hills, IL). Volatile fatty acids, lactate, formate, and ethanol were quantified by differential refractometry with a Waters HPLC (Waters Associates Inc., Milford, MA) equipped with a BioRad HPX 87H column (BioRad Laboratories, Hercules, CA). Solvent was 0.005 M H_2SO_4 at 0.6 mL/min. Column temperature was 65 °C. Sample injection volume was 15 µL. Crotonic acid co-eluted from the HPLC column with isovalerate. Consequently, isovalerate production is not reported. Ammonia concentration was determined as reported before (Chaney and Marbach, 1962).

Four replicates per compound and concentration were used. The experimental model was: response = control + linear effect of lumazine + quadratic effect of lumazine + crotonic acid effect + crotonic acid by linear effect of lumazine + crotonic acid by

quadratic effect of lumazine + residual. Non-significant ($P > 0.15$) quadratic terms were removed from the model. When significant ($P < 0.05$) interactions or tendencies ($P < 0.15$) were found, means were compared by Fisher least square differences (Rao, 1998). Significance was declared at $P < 0.05$.

Experiment 2

The experimental treatments of the 3 x 2 factorial arrangement were: 1) Lumazine 0 mM, 3-butenoic acid (Acros 15883 for all experiments) 0 mM (double control); 2) Lumazine 0 mM, 3-butenoic acid 4 mM; 3) Lumazine 0.6 mM, 3-butenoic acid 0 mM; 4) Lumazine 0.6 mM, 3-butenoic acid 4 mM; 5) Lumazine 1.2 mM, 3-butenoic acid 0 mM; 6) Lumazine 1.2 mM, 3-butenoic acid 4 mM. Lumazine was added as a solid, while 3-butenoic acid was added as a 1 mL-solution. 3-Butenoic acid controls received 1-mL of deionized water. Ruminal fluid collection and incubation, analytical procedures, and statistical analysis were done as described for Experiment 1, except that H_2 concentration could not be measured because of a malfunction of the detector. 3-Butenoic acid co-eluted from the HPLC column with propionate. Therefore, reported propionate production assume total disappearance of 3-butenoic acid.

Experiment 3

The 3 x 2 factorial arrangement of treatments included: 1) Propynoic acid (Acros 13150 for all experiments) 0 mM, crotonic acid 0 mM (double control); 2) Propynoic acid 0 mM, crotonic acid 4 mM; 3) Propynoic acid 2 mM, crotonic acid 0 mM; 4) Propynoic acid 2 mM, crotonic acid 4 mM; 5) Propynoic acid 4 mM, crotonic acid 0 mM;

6) Propynoic acid 4 mM, crotonic acid 4 mM. Both chemicals were added as 1 mL-solutions and controls received 1 mL of deionized water. Ruminal fluid collection and incubation, analytical procedures, and statistical analysis, were done as described for Experiment 1. Propynoic acid disappearance was also reported.

Experiment 4

The experimental treatments of the 3 x 2 factorial arrangement were: 1) Propynoic acid 0 mM, 3-butenoic acid 0 mM (double control); 2) Propynoic acid 0 mM, 3-butenoic acid 4 mM; 3) Propynoic acid 2 mM, 3-butenoic acid 0 mM; 4) Propynoic acid 2 mM, 3-butenoic acid 4 mM; 5) Propynoic acid 4 mM, 3-butenoic acid 0 mM; 6) Propynoic acid 4 mM, 3-butenoic acid 4 mM. Both chemicals were added as 1 mL-solutions and controls received 1 mL of deionized water. Ruminal fluid collection and incubation, analytical procedures, and statistical analysis were done as described for Experiment 1. Propynoic acid disappearance was also reported.

Experiment 5

The 3 x 2 factorial arrangement included: 1) Ethyl 2-butynoate (GFS Chemicals 3132 for all experiments) 0 mM, crotonic acid 0 mM (double control); 2) Ethyl 2-butynoate 0 mM, crotonic acid 4 mM; 3) Ethyl 2-butynoate 4 mM, crotonic acid 0 mM; 4) Ethyl 2-butynoate 4 mM, crotonic acid 4 mM; 5) Ethyl 2-butynoate 8 mM, crotonic acid 0 mM; 6) Ethyl 2-butynoate 8 mM, crotonic acid 4 mM. Ethyl 2-butynoate was added as a liquid, while crotonic acid was added as a 1 mL-solution. Crotonic acid controls received 1 mL of deionized water. Ruminal fluid collection and incubation,

analytical procedures, and statistical analysis were done as described for Experiment 1. Ethyl 2-butynoate co-eluted from the HPLC column with butyrate. Therefore, reported butyrate production assume total disappearance of ethyl 2-butynoate.

Experiment 6

The experimental treatments of the 3 x 2 factorial arrangement included: 1) Ethyl 2-butynoate 0 mM, 3-butenoic acid 0 mM (double control); 2) Ethyl 2-butynoate 0 mM, 3-butenoic acid 4 mM; 3) Ethyl 2-butynoate 4 mM, 3-butenoic acid 0 mM; 4) Ethyl 2-butynoate 4 mM, 3-butenoic acid 4 mM; 5) Ethyl 2-butynoate 8 mM, 3-butenoic acid 0 mM; 6) Ethyl 2-butynoate 8 mM, 3-butenoic acid 4 mM. Ethyl 2-butynoate was added as a liquid, while 3-butenoic acid was added as a 1 mL-solution. 3-Butenoic acid controls received 1 mL of deionized water. Ruminal fluid collection and incubation, analytical procedures, and statistical analysis were done as described for Experiment 1. Separation of crotonic acid from isovalerate was achieved by dropping the column temperature to 45 °C. Ethyl 2-butynoate was quantified by flame ionization detector with Perkin Elmer 8500 GC equipped with an AllTech AT-1 column. Solvent was ether at 4 mL/min flow rate. Start temperature was 80 °C. Ramp rate 1 was 10 °C/min. End temperature 1 was 150 °C. Ramp rate 2 was 30 °C/min, and end temperature 2 was 250 °C.

Results

Experiment 1

No interactions were found for CH_4 production. Lumazine decreased ($P < 0.01$) CH_4 production by 9 and 10% with and without crotonic acid, respectively (Table 6-1). Crotonic acid decreased ($P < 0.01$) methanogenesis by 10%. There were linear by linear and linear by quadratic interactions ($P < 0.01$) for CO_2 release. In the absence of crotonic acid, lumazine at 0.6 mM decreased ($P < 0.05$) CO_2 release by 18%; however, there were no effects in the presence of crotonic acid.

There were no interactions for H_2 and formate accumulation or ethanol production. No increase in H_2 accumulation was observed (Table 6-1). Unexpectedly, formate accumulation was strongly decreased ($P < 0.01$) by lumazine, while crotonic acid had no effect. None of the additives had an effect on ethanol production.

There was a linear by quadratic interaction for total VFA concentration ($P = 0.04$). In the absence of crotonic acid, lumazine did not affect total VFA concentration, while in its presence, lumazine at 0.6 mM decreased ($P < 0.05$) total VFA concentration. Crotonic acid increased ($P < 0.01$) total VFA concentration at 0 and 0.3 mM lumazine. There was a linear by quadratic interaction on acetate production ($P < 0.01$). Lumazine at 0.6 mM decreased ($P < 0.05$) acetate production with and without crotonic acid. Crotonic acid increased ($P < 0.01$) acetate production only at 0 and 0.3 mM lumazine. There was a tendency ($P = 0.07$) for a linear by quadratic interaction for propionate production. Lumazine decreased ($P < 0.01$) propionate production both in the absence and presence of crotonic acid. Crotonic acid decreased ($P < 0.05$) propionate production

Table 6-1. Effects of lumazine and crotonic acid on 24 h ruminal in vitro fermentation (Experiment 1)

Crotonic acid (mM)	0			8			Probabilities[1]					SEM
Lumazine (mM)	0	0.3	0.6	0	0.3	0.6	C	Lz	Lz[2]	C x Lz	C x Lz[2]	
CH_4 (µmol)	588	537	532	519	509	470	< 0.01	< 0.01	NS[2]	NS	NS	51.8
CO_2 (µmol)	2621[c,3]	2746[bc]	2139[d]	3187[a]	2948[ab]	2956[ab]	< 0.01	0.03	< 0.01	0.01	< 0.01	356
H_2 (µmol)	0.55	0.53	0.36	0.76	0.67	1.06	NS	NS	NS	NS	NS	1.48
Formate (µmol)	147	36.6	6.31	119	40.3	2.40	NS	< 0.01	0.06	NS	NS	35.5
Ethanol (µmol)	ND[4]	1.09	1.47	ND	ND	ND	NS	NS	NS	NS	NS	1.46
Total VFA (mM)	39.8[b]	39.6[b]	39.3[b]	42.6[a]	42.5[a]	40.0[b]	< 0.01	NS	NS	NS	0.04	1.30
Acetate (µmol)	2162[b]	2144[bc]	2096[c]	2276[a]	2255[a]	2112[bc]	< 0.01	0.01	NS	0.13	< 0.01	34.6
Propionate (µmol)	661[a]	633[b]	627[bc]	644[ab]	616[c]	559[d]	0.12	< 0.01	NS	NS	0.07	11.9
Butyrate (µmol)	148[c]	146[c]	149[c]	294[a]	299[a]	275[b]	< 0.01	NS	NS	NS	0.04	10.3
Isobutyrate (µmol)	12.2[d]	43.8[c]	68.7[a]	12.7[d]	48.1[c]	57.8[b]	NS	< 0.01	NS	< 0.01	< 0.01	4.52
Valerate (µmol)	66.4	67.4	69.5	63.3	64.5	63.1	NS	NS	NS	NS	NS	3.54
Final pH	7.0	7.1	6.9	7.1	7.0	6.9	NS	NS	NS	NS	NS	0.15
NH_4^+ (mg/100 mL)	25.4[b]	27.1[a]	26.3[ab]	25.7[b]	27.2[a]	25.3[b]	NS	< 0.01	< 0.01	0.08	NS	1.8

[1]C = crotonic acid, Lz = lumazine.

[2]NS = non-significant.

[3]Means with unlike superscripts in the same row differ ($P < 0.05$; Fisher LSD test).

[4]ND = not detected.

at 0.3 and 0.6 mM lumazine. There was a linear by quadratic interaction for butyrate production. In the absence of crotonic acid, lumazine did not affect butyrate production. However, butyrate production was decreased ($P < 0.05$) at 8 mM crotonic acid, lumazine and 0.6 mM lumazine. Crotonic acid increased ($P < 0.01$) butyrate production by about 2-fold. There were linear and linear by quadratic interactions ($P < 0.01$) for isobutyrate production. Lumazine at 0.3 and 0.6 mM increased ($P < 0.01$) isobutyrate production by 3.7- and 5.1-fold, respectively. Crotonic acid decreased ($P < 0.05$) isobutyrate production only at 0.6 mM lumazine. There were no interactions for final pH. Final pH was not affected by the additives. Ammonia concentration peaked ($P < 0.01$; quadratic response) at 0.3 mM lumazine.

Experiment 2

There was a linear by linear interaction ($P = 0.03$) for CH_4 production. Lumazine decreased ($P < 0.01$; Table 6-2) CH_4 production by 15 and 24 % with and without 3-butenoic acid, respectively. 3-Butenoic acid decreased ($P < 0.05$) CH_4 production only at 1.2 mM lumazine (linear interaction $P = 0.03$). There were no effects of the additives on CO_2 release.

No interactions for H_2 and formate accumulation or ethanol production. No increase in H_2 accumulation was observed (Table 6-2). Surprisingly, 3-butenoic acid increased formate accumulation, while lumazine had no effect. None of the additives influenced ethanol production.

There were no interactions for total VFA concentration or acetate, butyrate, valerate or isovalerate production. 3-Butenoic acid increased ($P < 0.01$; Table 6-2) total

Table 6-2. Effects of lumazine and 3-butenoic acid on 24 h ruminal in vitro fermentation (Experiment 2)

3-Butenoic acid (mM)	0			4			Probabilities[1]					SEM
Lumazine (mM)	0	0.6	1.2	0	0.6	1.2	B	Lz	Lz[2]	B x Lz	B x Lz[2]	
CH_4 (µmol)	405[a 2]	380[b]	342[c]	404[a]	367[b]	309[d]	NS[3]	< 0.01	NS	0.03	NS	31.2
CO_2 (µmol)	2049[a]	1826[a]	2105[a]	2262[a]	1836[a]	1755[a]	NS	NS	NS	0.14	NS	824
H_2 (µmol)	0.85	0.30	1.81	1.00	1.94	1.21	NS	NS	NS	NS	NS	3.99
Formate (µmol)	ND[4]	1.07	ND	31.6	24.4	34.7	< 0.01	NS	NS	NS	NS	6.71
Ethanol (µmol)	ND	1.06	ND	ND	0.73	1.01	NS	NS	NS	NS	NS	1.17
Total VFA (mM)	71.1	71.2	71.1	77.2	76.8	76.7	< 0.01	NS	NS	NS	NS	1.73
Acetate (µmol)	1474	1435	1388	1693	1639	1589	< 0.01	< 0.01	NS	NS	NS	22.6
Propionate (µmol)	449[a]	437[a]	422[b]	439[a]	409[b]	396[c]	0.03	< 0.01	NS	0.09	NS	8.69
Butyrate (µmol)	147	155	193	261	271	315	< 0.01	NS	< 0.01	NS	NS	8.68
Isobutyrate (µmol)	9.03[c]	66.5[b]	88.0[a]	10.1[c]	71.0[b]	83.3[a]	NS	< 0.01	< 0.01	NS	0.08	3.91
Valerate (µmol)	66.0	61.6	62.9	65.6	63.0	63.9	NS	NS	NS	NS	NS	3.25
Isovalerate (µmol)	37.9	29.9	30.2	34.4	29.3	30.5	NS	< 0.01	0.03	NS	NS	3.58
Final pH	6.81[ab]	6.81[ab]	6.86[a]	6.84[a]	6.73[c]	6.77[bc]	NS	0.14	NS	0.02	NS	0.11
NH_4^+ (mg/100 mL)	30.3	30.7	27.8	30.1	28.0	25.5	NS	NS	0.11	NS	NS	3.5

[1]B = 3-butenoic acid, Lz = lumazine.

[2]Means with unlike superscripts in the same row differ ($P < 0.05$; Fisher LSD test).

[3]NS = non-significant.

[4]ND = not detected.

VFA concentration, while lumazine did not affect it. Lumazine linearly decreased ($P < 0.01$) acetate production, while 3-butenoic acid increased ($P < 0.01$) it. There was a tendency ($P = 0.09$) for a linear by linear interaction for propionate production. Lumazine linearly decreased ($P < 0.01$) propionate production. 3-Butenoic acid decreased ($P < 0.05$) propionate production at 0.6 and 1.2 mM lumazine. Both lumazine and 3-butenoic acid increased butyrate production. Lumazine at 0.6 and 1.2 mM caused ($P < 0.01$) a 7- and 9-fold increase in isobutyrate production, respectively. It caused a quadratic decrease ($P = 0.03$) in isovalerate production and did not affect valerate. 3-Butenoic acid did not affect isobutyrate, valerate, or isovalerate production. There was a linear interaction for final pH ($P = 0.02$; Table 6-2). In the absence of 3-butenoic acid, lumazine did not affect pH, but it decreased it ($P < 0.05$) at 4 mM 3-butenoic acid. Lumazine at 1.2 mM tended ($P = 0.11$; quadratic response) to decrease ammonia concentration.

Experiment 3

There were no interactions for CH_4 production or CO_2 release. Both at 0 and 4 mM crotonic acid, propynoic acid decreased ($P < 0.01$; quadratic response) CH_4 production by 69% (Table 6-3). Crotonic acid decreased ($P = 0.02$) CH_4 production by 8, 19, and 9% at 0, 2, and 4 mM propynoic acid initial concentration, respectively. Release of CO_2 was linearly decreased ($P = 0.02$) by propynoic acid, but not affected by crotonic acid.

There was a linear by linear interaction ($P = 0.04$) for H_2 accumulation. Propynoic acid strongly increased ($P < 0.01$; quadratic response) H_2 accumulation. At 4

Table 6-3. Effects of propynoic acid and crotonic acid on 24 h ruminal in vitro fermentation (Experiment 3)

Crotonic acid (mM)	0			4			Probabilities[1]					
Propynoic acid (mM)	0	2	4	0	2	4	C	P	P^2	C x P	C x P^2	SEM
CH_4 (µmol)	536	270	170	493	220	155	0.02	< 0.01	< 0.01	NS[2]	NS	71.0
CO_2 (µmol)	2625	2373	1984	2448	2322	2261	NS	0.02	NS	NS	NS	676
H_2 (µmol)	ND[d 3,4]	28.4[c]	133[a]	ND[d]	37.8[c]	111[b]	NS	NS	< 0.01	0.04	NS	18.5
Formate (µmol)	20.4[b]	-7.72[c]	69.6[a]	-1.55[c]	22.2[b]	54.9[a]	0.02	< 0.01	< 0.01	< 0.01	< 0.01	11.9
Ethanol (µmol)	ND	5.98	14.8	ND	6.58	14.4	NS	< 0.01	NS	NS	NS	4.75
Propynoic acid disappearance (%)	-	10.2	8.29	-	7.65	7.18	NS	NS	-	NS	-	2.88
Total VFA (mM)	51.6	47.6	45.4	53.2	48.9	46.8	< 0.01	< 0.01	< 0.01	NS	NS	1.14
Acetate (µmol)	1683	1333	1202	1735	1356	1234	0.02	< 0.01	< 0.01	NS	NS	27.6
Propionate (µmol)	499[b]	540[a]	551[a]	482[c]	547[a]	545[a]	0.04	< 0.01	0.03	0.04	0.06	10.3
Butyrate (µmol)	156	167	130	263	258	217	< 0.01	0.04	< 0.01	NS	NS	13.4
Isobutyrate (µmol)	19.1[a]	8.29[c]	2.85[e]	16.2[b]	7.35[cd]	4.83[de]	0.02	< 0.01	< 0.01	0.02	NS	1.83
Valerate (µmol)	66.5[a]	36.7[b]	15.8[e]	60.8[a]	34.6[b]	22.2[c]	NS	< 0.01	NS	0.09	NS	6.79
Final pH	6.9[a]	6.8[bc]	6.7[cd]	6.8[ab]	6.6[d]	6.7[bc]	NS	NS	NS	0.07	0.04	0.14
NH_4^+ (mg / 100 mL)	26.0	22.5	21.9	26.1	22.8	22.3	NS	< 0.01	< 0.01	NS	NS	1.37

[1]C = crotonic acid, P = propynoic acid.
[2]NS = non-significant.
[3]Means with unlike superscripts in the same row differ ($P < 0.05$; Fisher LSD test).
[4]ND = not detected.

mM propynoic acid, crotonic acid decreased ($P < 0.05$) H_2 accumulation (linear interaction $P = 0.04$). There were also linear and linear by quadratic interactions ($P < 0.01$) on formate production (Table 6-3). In the absence of crotonic acid, accumulation of formate was minimal (and negative) at 2 mM propynoic acid, but increased by more than 3-fold of control levels at 4 mM propynoic acid ($P < 0.05$). At 4 mM crotonic acid, formate accumulation was increased ($P < 0.05$) by 2 and 4 mM propynoic acid. Crotonic acid decreased ($P < 0.05$) formate accumulation at 0 and 4 mM propynoic acid, but increased ($P < 0.05$) it at 2 mM. There were no interactions for ethanol production. Production of ethanol was linearly increased ($P < 0.01$) by propynoic acid and not affected by crotonic acid.

Little propynoic acid disappeared during the incubation period and this was not affected by its initial concentration or the presence of crotonic acid (Table 6-3).

There were no interactions for total VFA concentration. Crotonic acid increased ($P < 0.01$) total VFA concentration, while propynoic acid decreased ($P < 0.01$; quadratic response; Table 6-3) it. There were no interactions for acetate production. Propynoic acid decreased ($P < 0.01$; quadratic response) acetate production, while crotonic acid increased ($P = 0.02$) it. There was a linear by linear interaction ($P = 0.04$) and a tendency ($P = 0.06$) for a linear by quadratic interaction for propionate production. Propynoic acid increased ($P = 0.03$; quadratic response) propionate production. Crotonic acid decreased ($P < 0.05$) propionate production at 0 mM propynoic acid and not affected it at 2 and 4 mM propynoic acid. There were no interactions for butyrate production. Propynoic acid decreased ($P < 0.01$; quadratic response) butyrate production, and crotonic acid increased ($P < 0.01$) it. There was a linear by linear interaction ($P = 0.02$) for isobutyrate

production. Isobutyrate production was decreased ($P < 0.01$; quadratic response) by propynoic acid. Crotonic acid decreased ($P < 0.05$) isobutyrate production only in the absence of propynoic acid. Valerate production was decreased ($P < 0.01$) by propynoic acid, and not affected by crotonic acid. In the absence of crotonic acid, propynoic acid decreased ($P < 0.05$) final pH. There was a tendency ($P = 0.07$) for a linear by linear interaction, and a linear by quadratic interaction ($P = 0.04$) for final pH. At 4 mM crotonic acid, the lowest pH was observed at 2 mM propynoic acid ($P < 0.05$). Ammonia concentration was decreased ($P < 0.01$; quadratic response) by propynoic acid.

Experiment 4

There were linear by linear and linear by quadratic interactions for CH_4 production. Propynoic acid decreased ($P < 0.01$) CH_4 production by approximately 69% at both concentrations of 3-butenoic acid (Table 6-4). 3-Butenoic acid decreased ($P < 0.05$) CH_4 production only at 2 mM propynoic acid (linear and linear by quadratic interaction $P < 0.01$). There were no interactions for CO_2 release. Release of CO_2 was decreased ($P = 0.01$) by propynoic acid, and not affected by 3-butenoic acid.

There were linear by linear ($P = 0.01$) and linear by quadratic ($P = 0.04$) interactions for H_2 accumulation. Propynoic acid increased ($P < 0.01$; quadratic response) the accumulation of H_2. 3-Butenoic acid increased H_2 accumulation at 2 mM propynoic acid (Table 6-4). Propynoic acid increased formate ($P = 0.03$; quadratic relationship) accumulation. Unexpectedly, 3-butenoic acid also increased ($P < 0.01$) formate accumulation. Ethanol production was similar among treatments.

Table 6-4. Effects of propynoic acid and 3-butenoic acid on 24 h ruminal in vitro fermentation (Experiment 4)

3-Butenoic acid (mM)	0			4			Probabilities[1]					
Propynoic acid (mM)	0	2	4	0	2	4	B	P	P^2	B x P	B x P^2	SEM
CH_4 (µmol)	$487^{a\,2}$	316^{b}	155^{d}	508^{a}	221^{c}	156^{d}	NS[3]	< 0.01	NS	< 0.01	< 0.01	49.8
CO_2 (µmol)	1983	1760	1389	2245	1912	1997	NS	0.01	NS	NS	NS	668
H_2 (µmol)	$ND^{d\,4}$	48.2^{c}	74.0^{a}	ND^{d}	60.9^{b}	79.8^{a}	NS	< 0.01	< 0.01	0.01	0.04	10.2
Formate (µmol)	ND^{c}	ND^{c}	87.1^{b}	74.0^{b}	113^{b}	161^{a}	< 0.01	NS	0.03	NS	0.14	29.4
Ethanol (µmol)	ND	ND	ND	ND	ND	ND	NS	NS	NS	NS	NS	-
Propynoic acid disappearance (%)	-	6.43	4.26	-	3.90	2.52	NS	NS	-	NS	-	3.51
Total VFA (mM)	67.6	62.4	60.2	71.9	67.6	65.1	< 0.01	< 0.01	NS	NS	NS	4.9
Acetate (µmol)	1687	1423	1268	1820	1534	1417	0.08	< 0.01	0.14	NS	NS	228
Propionate (µmol)	523^{bc}	517^{c}	576^{a}	516^{c}	550^{ab}	551^{ab}	NS	NS	0.03	0.04	0.02	46.9
Butyrate (µmol)	127	159	172	257	295	304	< 0.01	0.02	NS	NS	NS	55.5
Isobutyrate (µmol)	6.5	3.2	6.6	-4.5	4.8	11.8	NS	NS	NS	NS	NS	30.7
Valerate (µmol)	62.1^{a}	38.2^{b}	1.87^{d}	54.8^{a}	36.2^{b}	17.0^{c}	0.15	< 0.01	NS	0.03	NS	21.6
Isovalerate (µmol)	40.2^{a}	21.0^{bc}	15.4^{cd}	33.1^{ab}	25.0^{bc}	6.8^{d}	NS	0.02	NS	0.13	0.11	17.2
Final pH	6.71	6.70	6.68	6.68	6.61	6.59	NS	NS	NS	NS	NS	0.13
NH_4^+ (mg/100 mL)	42.9	36.7	32.8	40.3	34.1	30.8	< 0.01	< 0.01	0.04	NS	NS	3.1

[1]B = 3-butenoic acid, P = propynoic acid; [2]Means with unlike superscripts in the same row differ ($P < 0.05$; Fisher LSD test); [3]NS = non-significant; [4]ND = not detected.

Little propynoic acid disappeared during the incubation period. Disappearance was not affected by its initial concentration or the presence of 3-butenoic acid (Table 6-4).

There were no interactions for total VFA concentration. Propynoic acid decreased ($P < 0.01$; Table 6-4) total VFA, while crotonic acid increased ($P < 0.01$) it. There were no interactions for acetate production. Acetate production was decreased ($P < 0.01$) by propynoic acid, and tended ($P = 0.08$) to increase with 3-butenoic acid. There were linear by linear ($P = 0.04$) and linear by quadratic ($P = 0.02$) interactions for propionate production. In the absence of 3-butenoic acid, propionate production was increased ($P < 0.05$) by propynoic acid only at 4 mM. At 4 mM 3-butenoic acid, propionate production was increased ($P < 0.05$) by both concentrations of propynoic acid. There were no interactions for butyrate or isobutyrate production. Butyrate production was increased by both propynoic acid ($P = 0.02$) and 3-butenoic acid ($P < 0.01$). None of the additives affected isobutyrate production. There was a linear interaction ($P = 0.03$) for isovalerate production. Both at 0 ($P = 0.02$; quadratic response) and 4 mM 3-butenoic acid, propynoic acid strongly decreased ($P < 0.01$) valerate production. 3-Butenoic acid increased ($P < 0.05$) valerate production at 4 mM propynoic acid. Propynoic acid decreased ($P = 0.02$) isovalerate production. There were no interactions for final pH or NH_4^+ concentration. Final pH was not affected by the additives. Ammonia concentration was decreased ($P < 0.01$) by both additives.

Experiment 5

There were no interactions for CH_4 production or CO_2 release. Ethyl 2-butynoate linearly decreased ($P < 0.01$) CH_4 production by more than 90% at both concentrations of crotonic acid. Crotonic acid did not affect methanogenesis (Table 6-5). Ethyl 2-butynoate decreased ($P < 0.01$) CO_2 release by 39%, while crotonic acid did not affect it.

There was a linear by linear interaction ($P = 0.04$) and a tendency ($P = 0.06$) for a linear by quadratic interaction for H_2 accumulation. Ethyl 2-butynoate increased the accumulation of H_2 ($P < 0.01$; quadratic response; Table 6-5). Dihydrogen accumulation was decreased ($P < 0.05$) by crotonic acid only at 4 mM ethyl 2-butynoate. There was a tendency ($P < 0.15$) for a linear by quadratic interaction for formate accumulation. Ethyl 2-butynoate increased ($P < 0.01$; quadratic response) formate accumulation. Crotonic acid increased ($P < 0.05$) formate accumulation at 8 mM ethyl 2-butynoate. There were no interactions for ethanol production. Ethyl 2-butynoate increased ($P < 0.01$; quadratic response) ethanol production, while crotonic acid did not affect it.

There were no interactions for total VFA concentration or acetate production. Ethyl 2-butynoate decreased ($P < 0.01$; quadratic response) total VFA concentration, while crotonic acid did not affect it (Table 6-5). Ethyl 2-butynoate decreased ($P < 0.01$; quadratic response) acetate production, while crotonic acid did not affect it. There were tendencies for linear by linear ($P = 0.13$) and linear by quadratic ($P = 0.11$) interactions for propionate production. At 0 mM crotonic acid, propionate production was greatest ($P < 0.05$) at 4 mM ethyl 2-butynoate. At 4 mM crotonic acid, propionate production was not affected by 4 mM ethyl 2-butynoate, and decreased ($P < 0.05$) by 8 mM ethyl 2-butynoate. Crotonic acid decreased ($P < 0.05$) propionate production only at 4 mM ethyl

Table 6-5. Effects of ethyl 2-butynoate and crotonic acid on 24 h ruminal in vitro fermentation (Experiment 5)

Crotonic acid (mM)	0			4			Probabilities[1]					
Ethyl 2-butynoate (mM)	0	4	8	0	4	8	C	EB	EB²	C x EB	C x EB²	SEM
CH_4 (μmol)	656	356	58.0	643	358	50.3	NS[2]	< 0.01	NS	NS	NS	38.1
CO_2 (μmol)	3286	2959	1908	3528	2714	2262	NS	< 0.01	NS	NS	NS	368
H_2 (μmol)	7.10[d 3]	36.1[b]	47.2[a]	6.71[d]	28.1[c]	43.7[a]	NS	< 0.01	< 0.01	0.04	0.06	3.88
Formate (μmol)	2.26[d]	174[c]	485[b]	5.93[d]	199[c]	593[a]	NS	< 0.01	< 0.01	NS	< 0.15	23.2
Ethanol (μmol)	ND[4]	296	470	ND	210	436	NS	< 0.01	< 0.15	NS	NS	40.3
Total VFA (mM)	73.2	68.9	55.7	74.9	69.3	56.8	NS	NS	< 0.01	NS	NS	2.52
Acetate (μmol)	2395	2014	1416	2422	2064	1448	NS	< 0.01	< 0.01	NS	NS	68.9
Propionate (μmol)	699[b]	781[a]	570[c]	680[b]	696[b]	556[c]	NS	< 0.01	< 0.01	0.13	0.11	46.8
Butyrate (μmol)	239[c]	243[c]	285[b]	327[a]	345[a]	341[a]	< 0.01	NS	0.10	NS	0.07	18.4
Isobutyrate (μmol)	34.8	102	211	31.3	63.5	198	NS	< 0.01	NS	NS	NS	19.0
Valerate (μmol)	80.8	80.7	36.6	76.6	73.7	34.8	NS	< 0.01	< 0.01	NS	NS	6.33
Final pH	6.8	6.7	6.8	6.7	6.7	6.8	NS	0.03	< 0.01	NS	NS	0.11
NH_4^+ (mg/100 mL)	34.9	27.1	26.1	31.7	26.9	25.2	0.08	< 0.01	0.01	NS	NS	4.64

[1]C = crotonic acid, EB = ethyl 2-butynoate.

[2]NS = non-significant.

[3]Means with unlike superscripts in the same row differ ($P < 0.05$; Fisher LSD test).

[4]ND = not detected.

2-butynoate. There was a tendency ($P = 0.07$) for a linear by quadratic interaction for butyrate production. Butyrate production was increased ($P < 0.01$) by crotonic acid. In the absence of crotonic acid, butyrate production was increased by 8 mM ethyl 2-butynoate. There were no interactions for isobutyrate and valerate production, final pH and NH_4^+ concentration. Isobutyrate production was increased ($P < 0.01$) by ethyl 2-butynoate, and not affected by crotonic acid. Valerate production was decreased ($P < 0.01$; quadratic response) by ethyl 2-butynoate, and not affected by crotonic acid. Effects of ethyl 2-butynoate on final pH ($P < 0.01$; quadratic response) were of little importance. Ammonia concentration was decreased ($P = 0.01$; quadratic response) by ethyl 2-butynoate and tended ($P = 0.08$) to be decreased by crotonic acid.

Experiment 6

There was a linear by linear interaction ($P = 0.02$) and a tendency ($P = 0.05$) for a linear by quadratic interaction for CH_4 production. Ethyl 2-butynoate decreased ($P < 0.01$) CH_4 below the limits of detection (Table 6-6). Methane production was increased ($P < 0.05$) by 3-butenoic acid only in the absence of ethyl 2-butynoate. There were no interactions for CO_2 release. Ethyl 2-butynoate decreased ($P = 0.03$; quadratic response) CO_2 release, while 3-butenoic acid increased ($P = 0.04$) it.

There were no interactions for H_2 and formate accumulation and ethanol production. Ethyl 2-butynoate increased ($P < 0.01$; quadratic response; Table 6-6) the accumulation of H_2, which was not relieved by 3-butenoic acid. Ethyl 2-butynoate also increased the accumulation of formate ($P = 0.01$; quadratic response) and ethanol production ($P < 0.01$), none of which was relieved by 3-butenoic acid.

Table 6-6. Effects of ethyl 2-butynoate and 3-butenoic acid on 24 h ruminal in vitro fermentation (Experiment 6)

3-Butenoic acid (mM)	0			4			Probabilities[1]					
Ethyl 2-butynoate (mM)	0	4	8	0	4	8	B	EB	EB^2	B x EB	B x EB^2	SEM
CH_4 (μmol)	493^{b}[2]	271^{c}	ND^{d}[3]	525^{a}	252^{c}	ND^{d}	0.03	< 0.01	0.05	0.02	0.05	40.4
CO_2 (μmol)	1682	1895	1252	2239	2187	1607	0.04	NS[4]	0.03	NS	NS	752
H_2 (μmol)	ND	7.2	36.2	ND	14.2	42.2	NS[3]	NS	< 0.01	NS	NS	10.1
Formate (μmol)	1.82	477	896	21.0	494	907	NS	< 0.01	0.01	NS	NS	24.1
Ethanol (μmol)	-0.71	132	291	-0.56	118	273	NS	< 0.01	NS	NS	NS	19.9
Total VFA (mM)	64.3	58.3	51.7	69.0	63.2	55.7	< 0.01	< 0.01	NS	NS	NS	2.87
Acetate (μmol)	1802^{b}	1503^{d}	1145^{f}	1980^{a}	1642^{c}	1264^{e}	< 0.01	< 0.01	NS	< 0.15	NS	40.1
Propionate (μmol)	621	621	513	584	617	497	0.06	< 0.01	< 0.01	NS	NS	23.0
Butyrate (μmol)	146^{e}	142^{e}	187^{d}	256^{c}	271^{b}	297^{a}	< 0.01	0.05	< 0.01	0.05	0.05	10.1
Isobutyrate (μmol)	27.6	45.4	154	24.0	46.3	153	NS	< 0.01	< 0.01	NS	NS	5.62
Valerate (μmol)	64.7	58.0	23.5	63.5	60.2	19.1	NS	0.05	< 0.01	NS	NS	6.30
Isovalerate (μmol)	31.3^{a}	6.36^{b}	3.38^{b}	34.3^{a}	0.99^{b}	6.24^{b}	NS	< 0.01	< 0.01	0.04	0.04	4.22
Final pH	6.85	6.83	6.87	6.81	6.78	6.82	NS	NS	NS	NS	NS	0.14
NH_4^+ (mg/100 mL)	24.1	20.4	20.7	23.7	17.2	19.9	NS	NS	NS	NS	NS	3.69

[1]B = 3-butenoic acid, EB = ethyl 2-butynoate.

[2]Means with unlike superscripts in the same row differ ($P < 0.05$; Fisher LSD test).

[3]ND = not detected.

[4]NS = non-significant.

Over 97% of ethyl 2-butynoate and 100% of 3-butenoic acid disappeared during fermentation (data not shown).

There were no interactions for total VFA, and a tendency ($P < 0.15$) for a linear by linear interaction for acetate production. Total VFA concentration and acetate production were decreased ($P < 0.01$) by ethyl 2-butynoate, and increased ($P < 0.01$) by 3-butenoic acid (Table 6-6). There were no interactions for propionate production. Propionate production was decreased ($P < 0.01$; quadratic response) by ethyl 2-butynoate at 8 mM, and tended ($P = 0.06$) to be decreased by 3-butenoic acid. Butyrate production was increased by both ethyl 2-butynoate ($P < 0.01$; quadratic response) and 3-butenoic acid ($P < 0.01$). There were no interactions for isobutyrate, valerate and isovalerate production, final pH and NH_4^+ concentration. Isobutyrate production was increased by ethyl 2-butynoate ($P < 0.01$; quadratic response), and not affected by 3-butenoic acid. Valerate and isovalerate production were decreased ($P < 0.01$; quadratic response) by ethyl 2-butynoate, and not affected by 3-butenoic acid. None of the additives affected the final pH or NH_4^+ concentration.

Discussion

Lumazine

In previous work, lumazine at 0.6 mM lowered CH_4 formation by 50%, and there was no additional reduction with 1.2 mM (Ungerfeld et al., 2002). Thus, a maximum initial concentration of 0.6 mM was chosen for Experiment 1. However, the inhibition achieved was substantially smaller than observed before (Ungerfeld et al., 2002). It was therefore decided to double the initial concentrations for Experiment 2. Nevertheless, the

inhibition of CH_4 formation was still lower than previously reported. Other responses have also been inconsistent or difficult to explain. In the previous study, acetate production was not affected even though CH_4 production was inhibited by 50% (Ungerfeld et al., 2002), while in Experiments 1 and 2 acetate production was decreased despite a much lower inhibition of methanogenesis. In Experiment 1 and in previous work (Ungerfeld et al., 2002), the acetate to propionate ratio increased (data not shown), which is contrary to the shift from acetate to propionate generally observed when CH_4 production is inhibited (Nagaraja et al., 1997). In Experiment 2, the acetate to propionate ratio was not affected by lumazine (data not shown). The observed increase in the acetate to propionate ratio, together with the decreased CO_2 production and lack of H_2 accumulation when methanogenesis was inhibited, led to the speculation that perhaps lumazine could somehow stimulate reductive acetogenesis (Ungerfeld et al., 2002). Tetrahydrofolate, like lumazine, a pterin derivative, is involved in reductive acetogenesis (Drake, 1994). Perhaps some acetogens could use lumazine to synthesize tetrahydrofolate.

Most puzzling, the addition of lumazine has resulted in large changes in isobutyrate production. A quadratic response, with a 10-fold decrease in isobutyrate production at 0.6 mM lumazine, and an almost complete recovery at 1.2 mM was first noticed (Ungerfeld et al., 2002). In Experiment 1, 4- to 5-fold increases were observed at 0.6 mM lumazine. In Experiment 2, 8- to 9-fold increases in isobutyrate production were observed at 1.2 mM lumazine. Isobutyrate is a product of valine fermentation (Voet and Voet, 1995). It is possible that lumazine somehow alters valine metabolism. Alternatively, isobutyrate may be a product of the catabolism of lumazine itself. In

Experiment 1, the C content of the extra isobutyrate represented 91 and 69% of the C present in lumazine, at 0.3 and 0.6 mM, respectively (data not shown). In Experiment 2, C in extra isobutyrate accounted for 129 and 83% of the C from added lumazine, at 0.6 and 1.2 mM, respectively (data not shown). However, as there are six C atoms per molecule of lumazine, and four per molecule of isobutyrate, one third of the C contained in lumazine should not appear in isobutyrate (assuming a molecule of isobutyrate is not formed from C provided from two lumazine molecules). This elevates the amount of lumazine necessary to form the extra isobutyrate to 137 and 104% of added lumazine at 0.3 and 0.6 mM lumazine in Experiment 1, and 194 and 125% of added lumazine at 0.6 and 1.2 mM lumazine in Experiment 2 (data not shown). Therefore, it seems unlikely that the increase in isobutyrate production was a result of lumazine catabolism.

In Experiment 1, formate production at 0 mM lumazine was unusually high (Table 6-1). Formate concentration in the double control (0 mM both additives) was 2.27 mM (data not shown), which is between about 100- and 200-fold higher than previously reported values (Hungate, 1967; Hungate et al., 1970; Asanuma et al., 1998). Formate concentration dropped by about 50 and 70% at 0.3 and 0.6 mM lumazine, respectively, but the absolute values appeared high compared to previously reported values. Highly fermentable substrates can lead to earlier and higher peaks of formate concentration in mixed ruminal batch cultures due to higher rates of formate production than utilization by methanogens (Asanuma et al., 1998). However, a slowly fermentable substrate (alfalfa hay) was used in the current experiments. Comparison of CH_4 production in Experiment 1 control treatment with controls in Experiments 2-6, would not support the premise that lower growth rates of methanogens in Experiment 1 resulted in formate accumulation.

Perhaps the utilization of formate as an electron donor for succinate (Asanuma et al., 1998) or butyrate formation was low in Experiment 1. Alternatively, more pyruvate may have been metabolized into formate by pyruvate formate lyase reaction (Russell and Wallace, 1997), or more formate may have been formed via glyoxylate (Asanuma et al., 1999).

Propynoic acid

Propynoic acid was again shown to be a fairly potent inhibitor of methanogenesis. As pointed out in Chapter 3, the inhibition of CH_4 production must not have been caused by competition for reducing equivalents because H_2 accumulation was consistently observed, and disappearance of propynoic acid was very low so that few reducing equivalents would be used to completely reduce its triple bond (Table 6-7). The effects on methanogenesis do not seem to be related to the amount of propynoic acid metabolized.

Propynoic acid addition caused an increase in the accumulation of H_2, formate, and ethanol in Experiment 3. However, and in contrast with previous observations (see Chapter 2), no accumulation of succinate was observed.

As it has been consistently reported, the inhibition of methanogenesis shifted the VFA profile from acetate to propionate (Nagaraja et al., 1997). However, the responses in butyrate production were different in Experiment 3 and 4. Propynoic acid decreased butyrate production in Experiment 3 but increased it in Experiment 4. The increase in butyrate production in Experiment 4 led to an increase ($P < 0.01$) in butyrate molar percentage from 7.5 to 10.8% (data not shown). As the procedures used in both experiments were the same, it is difficult to explain the opposite responses of butyrate

production to propynoic acid. A similar response was noted for isobutyrate production. In Experiment 3, propynoic acid strongly decreased isobutyrate by more than 4-fold, but it did not affected in Experiment 4. Both valerate and isovalerate production were strongly decreased by propynoic acid in both experiments. Isobutyrate, valerate, and isovalerate are fermentation products from valine, proline, and leucine, respectively (Nagaraja et al., 1997), and their decrease could reflect an inhibition of the fermentation of amino acids by propynoic acid. This agrees with the decline observed in NH_4^+ concentrations in both experiments. Decreased fermentation of amino acids has been reported when CH_4 formation was inhibited with CO (Russell and Martin, 1984). Deamination releases one pair of reducing equivalents per mole of NH_4^+ formed (Voet and Voet, 1995). The increase in H_2 partial pressure that occurs when methanogenesis is inhibited, results in an inhibition of deamination, especially for highly reduced, branched-chain amino acids (Hino and Russell, 1985).

Table 6-7. Proportion of the decrease in CH_4 formation accountable by the complete reduction of propynoic acid triple bond (Experiments 3 and 4)

Experiment	Initial concentration (mM)	Second additive (mM)	Pairs of 2H potentially taken (μmoles)	Decrease in CH_4 accountable by hydrogenation (%)
3	2	Crotonic acid 0 mM	33.4	3.14
3	2	Crotonic acid 4 mM	25.1	2.30
3	4	Crotonic acid 0 mM	54.4	3.73
3	4	Crotonic acid 4 mM	47.1	3.48
4	2	3-Butenoic acid 0 mM	13.4	1.96
4	2	3-Butenoic acid 4 mM	8.10	0.71
4	4	3-Butenoic acid 0 mM	17.7	1.33
4	4	3-Butenoic acid 4 mM	10.5	0.75

Ethyl 2-butynoate

Ethyl 2-butynoate was again shown to be a potent inhibitor of methanogenesis. Carbon dioxide release was also decreased, as shown before (Chapter 3).

In both Experiment 5 and 6, and as observed before (Chapter 3), ethyl 2-butynoate increased H_2, formate, and ethanol accumulation; indicating that the electrons spared from CH_4 formation were not efficiently relocated into normal end products of fermentation like propionate or butyrate.

In both Experiment 5 and 6, the acetate to propionate ratio was decreased ($P < 0.01$; quadratic response; data not shown), which is a normal consequence of the inhibition of ruminal methanogenesis (Nagaraja et al., 1997). Butyrate production was increased by ethyl 2-butynoate in both experiments, acting a H sink for part of the reducing equivalents spared from CH_4 formation. In agreement with previous results (Chapter 3), isobutyrate production was greatly increased in both experiments. This could be a consequence of a greater deamination of valine (Nagaraja et al., 1997). However, NH_4^+ concentration was decreased in Experiment 5, and this may suggest a general decrease in amino acid fermentation. Similarly, valerate and isovalerate, which are products of proline and leucine catabolism, respectively (Nagaraja et al., 1997), were decreased. Alternatively, isobutyrate could be a product of ethyl 2-butynoate metabolism. The C in extra isobutyrate represented 6.5 and 20.7% of ethyl 2-butynoate C in Experiment 6, where all the additive disappeared. In Experiment 5, it represented 16.3 and 29.7% of C in ethyl 2-butynoate. Then, isobutyrate increase could have been the result of ethyl 2-butynoate catabolism.

The accumulation of formate and ethanol when methanogenesis was inhibited by ethyl 2-butynoate was considerably greater than with propynoic acid. On the contrary, more H_2 accumulated with propynoic acid than with ethyl 2-butynoate. As almost all of ethyl 2-butynoate, but little propynoic acid, was metabolized by the mixed ruminal microbiota, it is possible that formate and/or ethanol were products of ethyl 2-butynoate catabolism.

Crotonic acid

As observed in Chapter 3, crotonic acid caused a mild decrease in CH_4 formation in Experiments 1 and 3, although no effect was observed in Experiment 5. It was hypothesized that crotonic acid would serve as an electron acceptor. This would rechannel electrons spared from methanogenesis into butyrate formation, and decrease the accumulation of reduced products without a nutritional value, such as H_2, formate, and ethanol. In Experiment 1, the addition of 656 μmoles of crotonate resulted in only 142 μmoles of extra butyrate (22%). It was thought that decreasing the initial concentration of crotonic acid to 4 mM could increase its efficiency of conversion into butyrate. However, in Experiment 3, crotonic acid did not relieve the increase in H_2 accumulation or ethanol production caused by propynoic acid and caused a small decrease in formate accumulation at 4 mM propynoic acid. Part of crotonic acid was converted to butyrate as hypothesized, and smaller amounts were converted to acetate. Averaged across propynoic acid concentrations, the addition of 328 μmoles of crotonic acid resulted in an increase in butyrate production of 95 μmoles (29%). The reduction of crotonate to butyrate would imply an uptake of 95 μmoles reducing equivalent pairs

(Miller and Jenesel, 1979). The increase in acetate production, 36 μmoles, would imply a release of 18 μmoles of reducing equivalent pairs (Miller and Jenesel, 1979; Russell, 2002), therefore not compensating for the metabolic H uptake needed for butyrate formation. As the effects on other VFA were minor, it is not clear why in Experiment 3 the uptake of metabolic H required for the conversion of crotonic acid into butyrate did not result in greater decreases of fermentation end products without a nutritional value.

In Experiment 5, crotonic acid caused some decrease in H_2 accumulation and ethanol production. Forty percent of added crotonic acid seemed to be converted to butyrate, while smaller amounts seemed to be converted to acetate. There was an 82 μmoles increase in butyrate production, a numerical increase in acetate of 36 μmoles, and a numerical decrease in propionate of 40 μmoles. Effects on other VFA were minor. The increase in acetate would imply a release of 18 μmoles reducing equivalents pairs, and the decrease in propionate formation would mean that 80 μmoles reducing equivalent pairs were not taken up by this pathway (Russell, 2002). Changes in acetate and propionate, when considered together, could compensate for the 82 μmoles reducing equivalents pairs incorporated into butyrate production. This could explain why the decrease in unusual reduced end products of fermentation was not more pronounced.

3-Butenoic acid

There was no accumulation of unusual fermentation products in Experiment 2. In Experiment 4, 3-butenoic acid did not decrease H_2 accumulation caused by propynoic acid, and, unexpectedly, increased formate accumulation. 3-Butenoic acid increased

butyrate production by 133 μmoles, but also increased acetate production by 131 μmoles. Effects on other VFA were minor. Increased acetate production would imply a release of 66 μmoles of reducing equivalent pairs, while the increase in butyrate would need 133 μmoles reducing equivalent pairs (Russell, 2002). In total, these changes would imply an uptake of approximately 67 μmoles of reducing equivalents pairs. However, there was no effect on unusual reduced end products. In Experiment 6, 3-butenoic acid was again ineffective in decreasing H_2, formate, or ethanol. The increase in acetate production (145 μmoles) was slightly higher than the corresponding increase in butyrate (116 μmoles). These changes, together with a tendency ($P = 0.06$) to decrease propionate production by 19 μmoles, would result in almost no change caused by 3-butenoic acid on the H balance (145 μmoles/2 - (-19 μmoles x 2 – 116 μmoles = -5.5 μmoles). This would explain the lack of effect of 3-butenoic acid in capturing reducing equivalents, and withdrawing them from CH_4 formation, or H_2, formate or ethanol accumulation.

The lack of effect of crotonic acid and 3-butenoic acid on decreasing the accumulation of H_2, formate and ethanol can be explained by: a) incomplete disappearance of the additives, and b) alternative metabolic pathways. With the exception of Experiment 6, additive disappearance was not measured due to additives co-eluting from the HPLC column with VFA. Significant amounts of both crotonic acid and 3-butenoic acid were metabolized to acetate. Likely, this follows the reversal of butyrate formation in the rumen, leading to the formation of two moles of acetyl CoA (Miller and Jenesel, 1979), and, eventually, acetate. Metabolism of intermediates of fermentation by the reverse of their normal pathways has been observed before (Chapter 3). The reason for a compound being metabolized in an opposite direction than normal can be

understood on thermodynamic grounds. A large increase in the concentration of a metabolic intermediate normally found at very low concentrations would make alternative metabolic pathways thermodynamically feasible, including the reverse of the normal pathway. It follows that to maximize the efficiency of conversion of crotonic acid or 3-butenoic acid into butyrate, these compounds should be added to the fermentation continuously, avoiding large increases in their concentrations.

Conclusions

Propynoic acid and ethyl 2-butynoate were again shown to be potent inhibitors of CH_4 production. Inhibition of methanogenesis resulted in inefficient relocation of electrons into H_2, formate, and ethanol. Neither crotonic acid nor 3-butenoic acid were effective in re-channeling the electrons spared from methanogenesis into butyrate formation. This was partly due to the fact that significant amounts of both additives were converted to acetate, a process that releases reducing equivalents. The relatively high concentrations of the additives may have made this alternative pathway thermodynamically feasible. Possibly, a more continuous supply of these additives would increase the proportion converted to butyrate and improve the uptake of reducing equivalents.

The decreases in methanogenesis caused by lumazine were lower than previously reported. The changes in the VFA profile were also erratic. The inconsistent effects of lumazine across experiments on in vitro ruminal fermentation are difficult to explain. The effects of lumazine on pure cultures of methanogens and other ruminal

microorganisms would need to be evaluated in order to understand its variable effects on mixed ruminal cultures.

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CHAPTER 7

Effects of combinations of inhibitors of methanogenesis with crotonic acid or 3-butenoic acid on in vitro degradation and microbial biomass and N synthesis

Abstract

It was hypothesized that the combinations of the methanogenesis inhibitors lumazine, propynoic acid, and ethyl 2-butynoate, with crotonic acid or 3-butenoic acid would be able to relieve the fermentation constraints observed when the inhibitors of methanogenesis are used. In six experiments, one of the three methanogenesis inhibitors, at three different concentrations, were combined with either crotonic acid or 3-butenoic acid, at two different concentrations. A 4:1 buffer to ruminal fluid mixture was incubated with grass hay in 1 L Erlenmeyer flasks for 72 h. ^{15}N incorporation was used as a microbial marker. All three methanogenesis inhibitors decreased N degradation. Propynoic acid and ethyl 2-butynoate at the highest concentration, also decreased OM degradation. Crotonic acid and 3-butenoic acid were ineffective to increase degradation of OM or N. All three inhibitors increased the production of microbial OM and N. Propynoic acid increased the amount of microbial OM and N per kilogram of total OM disappeared. Propynoic acid disappearance was very small. Ethyl 2-butynoate, and perhaps lumazine, could have been used as C and energy sources. Propynoic acid increased the proportion of total disappeared OM (substrate plus additive) that was incorporated into microbial biomass. It might have increased the efficiencies of microbial OM and N synthesis by decreasing bacterial cell lysis. It was concluded that

lumazine and ethyl 2-butynoate at low concentrations could benefit animal production by lowering CH_4 formation, decreasing proteolysis, and increasing microbial N flow.

Introduction

Methane is formed in the rumen as an end product of fermentation and is removed by eructation. Its release represents a loss of digestible energy for ruminants. Methane is also a potent greenhouse gas that contributes to global warming (Moss, 1993). There would be environmental and economic benefits in decreasing CH_4 production in the rumen. In previous work, lumazine, propynoic acid, and ethyl 2-butynoate were shown to inhibit ruminal methanogenesis (Ungerfeld et al., 2000, 2002). Unfortunately, apparent OM fermented, as estimated through a mass balance, was decreased.

In contrast, crotonic acid and 3-butenoic acid had little effects on methanogenesis, but seemed to stimulate OM fermentation (Ungerfeld et al., 2000). The objectives of this series of six experiments were to study the effects of combinations of lumazine, propynoic acid, and ethyl 2-butynoate with crotonic acid or 3-butenoic acid on mixed ruminal cultures. It was hypothesized that the combinations of inhibitors of CH_4 formation with crotonic acid or 3-butenoic acid would be able to lift the constraints on fermentation while maintaining the inhibition of methanogenesis.

Materials and Methods

Experiment 1

The experimental treatments of the 3 x 2 factorial arrangement were: 1) Lumazine (Sigma L 0380 for all experiments) 0 mM, crotonic acid (Sigma C4630 for all experiments) 0 mM (double control); 2) Lumazine 0 mM, crotonic acid 8 mM; 3) Lumazine 0.3 mM, crotonic acid 0 mM; 4) Lumazine 0.3 mM, crotonic acid 8 mM; 5) Lumazine 0.6 mM, crotonic acid 0 mM; 6) Lumazine 0.6 mM, crotonic acid 8 mM. Ruminal fluid was collected prior to the morning feeding from two non-lactating Holstein cows fed grass hay. A mixture of 150 mL of ruminal fluid and 600 mL of buffer (Goering and Van Soest, 1975) was anaerobically delivered into 1 L-Erlenmeyer flasks. Each flask had 6 g of ground (1 mm screen mesh) grass hay (1.0% N, 67.1% NDF, 6.3% ash, DM basis). Lumazine was added as a solid (4 and 8 mg for 0.3 and 0.6 mM, respectively), while crotonic acid was added as 10 mL of a 0.308 M solution. Crotonic acid controls received 10 mL of deionized water. Three samples of the ruminal fluid and buffer mixture were frozen for subsequent determination of VFA initial concentrations. Flasks were sealed under an O_2-free CO_2 atmosphere with stoppers that allowed gas release but not entrance, and incubated in a non-shaking water bath at 39 °C for 72 h. An aliquot of 5 mL was taken every 24 h for subsequent determination of crotonic acid concentrations. Crotonic acid was quantified by differential refractometry with a Waters 712 Wisp HPLC (Waters Associates Inc., Milford, MA) equipped with a BioRad HPX 87H column (BioRad Laboratories, Hercules, CA). Solvent was 0.005 M H_2SO_4 at 0.6 mL/min. Column temperature was 45 °C. Sample injection volume was 15 μL.

At the end of the incubation period, flasks were opened, and pH measured under CO_2 (Digital Benchtop pH Meter, Cole-Parmer Instrument Company, Vernon Hills, IL). Ten milliliters of 10% (v/v) H_2SO_4 were added to stop the fermentation. Flasks were immediately refrigerated until further analysis.

Each flask content was removed and centrifuged at 500 x *g* at 4 °C for 15 min (Zinn and Owens, 1986). The supernatants were removed using a Pasteur pipette, and the pellets of undigested substrate with attached microbial biomass ("pellets A") transferred into pre-weighted aluminum pans using an 0.85% saline solution. Supernatants were centrifuged at 4,640 x *g* at 4 °C for 30 min (Hsu and Fahey, 1990). The supernatant from the second centrifugation was removed using a Pasteur pipette and discarded. The resulting pure microbial pellets ("pellets B") were re-suspended in an 0.85% saline solution and re-centrifuged at 4,640 x *g* at 4 °C for 30 min. Pellets suspended in saline were transferred into pre-weighted aluminum pans. Pans containing both low- and high-speed centrifugation pellets (A & B) were freeze-dried for 5 d (Tri-Philizer MP, FTS Kynetics, Stone Ridge, NY). Pellet masses were determined from difference in pans masses. Pellets were analyzed for ash (Van Soest and Robertson, 1985), total N by generic combustion (AOAC, 1990) in a FP-2000 analyzer (Leco®, St. Joseph, MI), and ^{15}N enrichment (Europa Integra mass spectrometer, PDZ Europa, Northwich Cheshire, UK; Stable Isotope Facility, University of California-Davis). Pellets A were also analyzed for NDF content (Van Soest et al., 1991). The three samples taken for initial VFA content were combined into one, and centrifuged at 4,640 x *g* at 4 °C for 30 min (Hsu and Fahey, 1990). The ^{15}N enrichment of the resulting pellet was defined as ^{15}N

natural abundance. The proportion of microbial N in the low-speed centrifugation pellet A (PMN_A) was calculated as:

PMN_A = (^{15}N% in pellet A – natural abundance) / (^{15}N in pellet B – natural abundance)

Total microbial N was calculated as the addition of the microbial N in pellets A and B. Microbial OM in pellet A was calculated using the N/OM ratio of pellet B, and total microbial OM produced was calculated as the sum of microbial OM in pellets A and B. Microbial C was calculated from the empirical formula $C_6H_{9.85}O_{2.99}N_{1.20}$ (Stanier and Davies, 1981). True degradation of N (N_{TD}) and OM (OM_{TD}) , and apparent NDF degradation (NDF_{AD}), were calculated from their disappearances during fermentation:

N_{TD} (%) = 100 x (substrate N - N in pellet A + microbial N in pellet A) / substrate N

OM_{TD} (%) =

100 x (substrate OM – OM in pellet A + microbial OM in pellet A) / substrate OM

NDF_{AD} (%) = 100 x (substrate NDF - NDF in pellet A) / substrate NDF

Efficiencies of microbial N and OM synthesis were expressed on a truly degraded substrate OM basis.

Degraded OM can either form fermentation products (VFA, gases, NH_4^+, etc) or be incorporated into microbial OM (Leng and Nolan, 1984). The proportion of total disappeared OM (substrate plus additives) incorporated into microbial OM (PMOM), was calculated as:

PMOM = microbial OM / (substrate degraded OM + OM disappeared from additives)

Three replicates per compound and concentration were used. The experimental model was: observation = overall mean + linear effect of lumazine + quadratic effect of lumazine + crotonic acid effect + crotonic acid by linear effect of lumazine + crotonic acid by quadratic effect of lumazine + residual. Non-significant ($P > 0.15$) quadratic terms were removed from the model. When significant ($P < 0.05$) interactions or tendencies ($P < 0.15$) were found, means were compared by Fisher least square differences (Rao, 1998). Significance was declared at $P < 0.05$.

Crotonic acid disappearance as a function of time was modeled as a logistic response (Neter et al., 1996) for each lumazine concentration:

$$\%\text{disappeared} = 100 \times \exp(\beta_0 + \beta_1 t) / [1 + \exp(\beta_0 + \beta_1 t)]$$

where t is time in hours.

Experiment 2

The experimental treatments of the 3 x 2 factorial arrangement were: 1) Lumazine 0 mM, 3-butenoic acid (Acros 15883 for all experiments) 0 mM (double control); 2) Lumazine 0 mM, 3-butenoic acid 4 mM; 3) Lumazine 0.6 mM, 3-butenoic acid 0 mM; 4) Lumazine 0.6 mM, 3-butenoic acid 4 mM; 5) Lumazine 1.2 mM, 3-butenoic acid 0 mM; 6) Lumazine 1.2 mM, 3-butenoic acid 4 mM. Ruminal fluid was collected, processed, and incubated, as described for Experiment 1. Lumazine was added as a solid (8 and 16 mg for 0.6 and 1.2 mM, respectively). 3-Butenoic acid was added as 10 mL of a 0.308 M

solution. 3-Butenoic acid controls received 10 mL of deionized water. Analytical and statistical procedures were similar to Experiment 1.

Experiment 3

The 3 x 2 factorial arrangement of treatments included: 1) Propynoic acid (Acros 13150 for all experiments) 0 mM, crotonic acid 0 mM (double control); 2) Propynoic acid 0 mM, crotonic acid 4 mM; 3) Propynoic acid 2 mM, crotonic acid 0 mM; 4) Propynoic acid 2 mM, crotonic acid 4 mM; 5) Propynoic acid 4 mM, crotonic acid 0 mM; 6) Propynoic acid 4 mM, crotonic acid 4 mM. Ruminal fluid was collected, processed, and incubated, as described for Experiment 1. Propynoic acid and crotonic acid were added as 0.156 (10 or 20 mL) and 0.312 M (10 mL) solutions, respectively. Volumes were equalized with deionized water. Analytical and statistical procedures were the same as in Experiment 1, except that sampling was only done at the end of the 72 h-period. Efficiencies of microbial N and OM synthesis were expressed both on a truly degraded substrate OM basis (substrate only) and on a truly degraded total OM (substrate plus additive) basis.

Propynoic acid disappearance after 72 h of incubation was modeled as a response to propynoic acid (2 or 4 mM) and crotonic acid (0 or 4 mM) concentration, and their interaction. Crotonic acid disappearance was modeled as a quadratic response to propynoic acid concentration.

Experiment 4

The experimental treatments of the 3 x 2 factorial arrangement were: 1) Propynoic acid 0 mM, 3-butenoic acid 0 mM (double control); 2) Propynoic acid 0 mM, 3-butenoic acid 4 mM; 3) Propynoic acid 2 mM, 3-butenoic acid 0 mM; 4) Propynoic acid 2 mM, 3-butenoic acid 4 mM; 5) Propynoic acid 4 mM, 3-butenoic acid 0 mM; 6) Propynoic acid 4 mM, 3-butenoic acid 4 mM. Ruminal fluid was collected, processed, and incubated, as described for Experiment 1. Propynoic acid and 3-butenoic acid were added as 0.156 (10 or 20 mL) and 0.312 M (10 mL) solutions, respectively. Volumes were equalized with deionized water. Analytical and statistical procedures were the same as in Experiment 3. Efficiencies of microbial N and OM synthesis were expressed both on a truly degraded substrate OM basis (substrate only) and on a truly degraded total OM (substrate plus additive) basis.Propynoic acid disappearance at 72 h was modeled as a response to propynoic acid (2 or 4 mM) and 3-butenoic acid (0 or 4 mM) concentrations, and the interaction.

Experiment 5

The 3 x 2 factorial arrangement included: 1) Ethyl 2-butynoate (GFS Chemicals 3132 for all experiments) 0 mM, crotonic acid 0 mM (double control); 2) Ethyl 2-butynoate 0 mM, crotonic acid 4 mM; 3) Ethyl 2-butynoate 4 mM, crotonic acid 0 mM; 4) Ethyl 2-butynoate 4 mM, crotonic acid 4 mM; 5) Ethyl 2-butynoate 8 mM, crotonic acid 0 mM; 6) Ethyl 2-butynoate 8 mM, crotonic acid 4 mM. Ruminal fluid was collected, processed, and incubated, as described for Experiment 1. Ethyl 2-butynoate was delivered as a liquid (0.35 and 0.70 mL for 4 and 8 mM, respectively), while crotonic

acid was added as 10 mL of a 0.304 M solution. Crotonic acid controls received equal volumes of deionized water. Analytical and statistical procedures were the same as in Experiment 1. Efficiencies of microbial N and OM synthesis were expressed both on a truly degraded substrate OM basis (substrate only) and on a truly degraded total OM (substrate plus additive) basis. As almost all ethyl 2-butynoate disappeared by 24 h, only data for that time period are presented for ethyl 2-butynoate disappearance. Ethyl 2-butynoate disappearance was modeled as a response to ethyl 2-butynoate (4 or 8 mM) and crotonic acid (0 or 4 mM) concentrations and the resulting interaction. Crotonic acid disappearance as a function of time was modeled as a logistic response (Neter et al., 1996) for each ethyl 2-butynoate concentration:

$$\%\text{disappeared} = 100 \times \exp(\beta_0 + \beta_1 t) / [1 + \exp(\beta_0 + \beta_1 t)]$$

where t is time in hours. Comparisons of crotonic acid disappearance among ethyl 2-butynoate concentrations at 24 h were done using the Tukey test (Neter et al., 1996).

Experiment 6

The experimental treatments of the 3 x 2 factorial arrangement included: 1) Ethyl 2-butynoate 0 mM, 3-butenoic acid 0 mM (double control); 2) Ethyl 2-butynoate 0 mM, 3-butenoic acid 4 mM; 3) Ethyl 2-butynoate 4 mM, 3-butenoic acid 0 mM; 4) Ethyl 2-butynoate 4 mM, 3-butenoic acid 4 mM; 5) Ethyl 2-butynoate 8 mM, 3-butenoic acid 0 mM; 6) Ethyl 2-butynoate 8 mM, 3-butenoic acid 4 mM. Ruminal fluid was collected, processed, and incubated, as described for Experiment 1. Ethyl 2-butynoate was delivered as a liquid (0.35 and 0.70 mL for 4 and 8 mM, respectively), while 3-butenoic acid was added as 10 mL of a 0.304 M solution. 3-Butenoic acid controls received equal

volumes of deionized water. Analytical and statistical procedures were the same as in Experiment 1. Efficiencies of microbial N and OM synthesis were expressed on a truly degraded substrate OM basis (substrate only) and on a truly degraded total OM (substrate plus additive) basis. As almost all ethyl 2-butynoate disappeared by 24 h, only data for that time period are presented for ethyl 2-butynoate disappearance. Ethyl 2-butynoate disappearance was modeled as a response to ethyl 2-butynoate (4 or 8 mM) and crotonic acid (0 or 4 mM) concentrations and the resulting interaction. 3-Butenoic acid disappearance as a function of time was modeled as a logistic response (Neter et al., 1996) for each ethyl 2-butynoate concentration:

$$\%\text{disappeared} = 100 \times \exp(\beta_0 + \beta_1 t) / [1 + \exp(\beta_0 + \beta_1 t)]$$

where t is time in hours.

Results

Experiment 1

Over 90% of crotonic acid disappeared after 48 h of incubation. However, substantially less crotonic acid had disappeared after 24 h of incubation, especially at 0.3 mM lumazine (Figure 7-1).

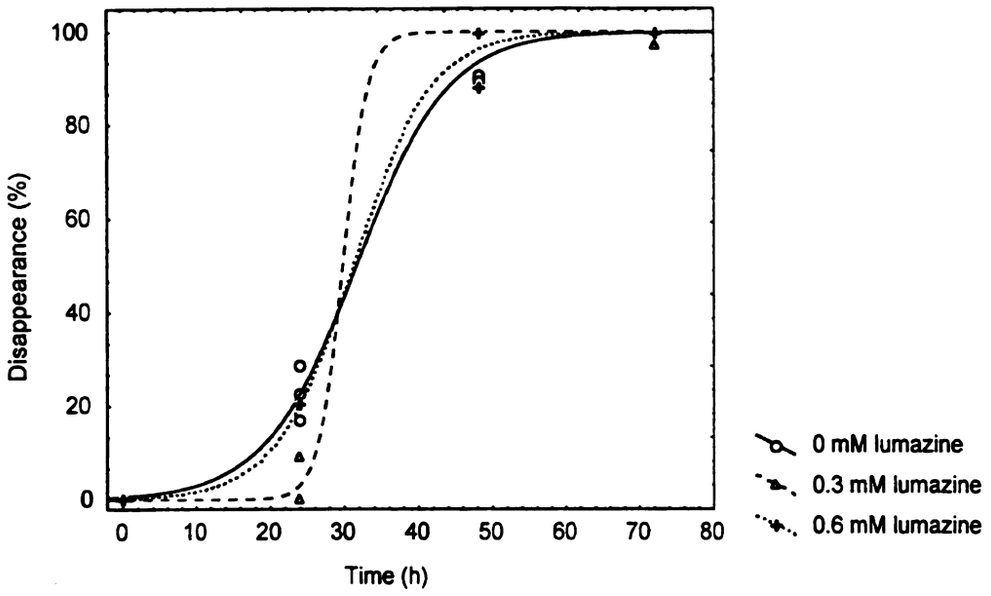

Figure 7-1 Crotonic acid disappearance (Experiment 1)

There were no interactions for OM degradation, and none of the additives affected it. There was a tendency for a linear by quadratic interaction ($P = 0.13$) for N degradation. Lumazine tended ($P = 0.06$; quadratic response; Table 7-1) to decrease N degradation. At 0 mM crotonic acid, N degradation was decreased ($P < 0.05$) by 0.3 mM lumazine, while at 4 mM crotonic acid, N degradation was decreased ($P < 0.05$) by 0.6 mM lumazine. Crotonic acid decreased ($P < 0.05$) N degradation at 0.6 mM lumazine. There were no interactions for apparent NDF degradation, and none of the additives affected it. There were linear by linear and linear by quadratic interactions ($P = 0.04$) for the proportion of OM incorporated into microbial biomass. In the absence of crotonic acid, lumazine at 0.3 mM increased ($P < 0.05$) the proportion of OM incorporated into microbial biomass, while there was no effect of lumazine at 8 mM crotonic acid. There were tendencies for linear by linear ($P = 0.06$) and linear by quadratic ($P = 0.09$) interactions for microbial OM production. At 0 mM crotonic acid, lumazine at 0.3 and

Table 7-1. Effects of lumazine and crotonic acid on 72 h in vitro degradation and microbial biomass production (Experiment 1)

Crotonic acid (mM)	0			8			Probabilities[1]					SEM[2]
Lumazine (mM)	0	0.3	0.6	0	0.3	0.6	C	Lz	Lz^2	C x Lz	C x Lz^2	
True OM degradation (%)	41.5	38.0	39.6	41.7	43.7	40.1	NS[6]	NS	NS	NS	NS	2.80
True N degradation (%)	54.2^{a}	48.6^{bc}	49.5^{ab}	50.0^{ab}	48.5^{bc}	44.1^{c}	0.10	0.06	0.14	NS	0.13	2.92
Apparent NDF degradation (%)	40.4	42.1	38.5	41.7	42.2	38.0	NS	NS	NS	NS	NS	2.70
PMOM[3]	0.23^{bc}	0.27^{a}	0.26^{ab}	0.20^{cd}	0.19^{d}	0.21^{cd}	0.11	0.05	0.10	0.04	0.04	0.021
Microbial OM (mg)	522^{c}	569^{ab}	578^{ab}	563^{abc}	546^{bc}	592^{a}	0.09	0.01	NS	0.06	0.09	24.4
Microbial N (mg)	47.6	51.0	55.3	50.2	53.7	57.9	< 0.05	< 0.01	NS	NS	NS	1.63
EMOMS[4] (g of microbial OM /kg of degraded substrate OM)	231^{b}	276^{a}	268^{ab}	248^{ab}	229^{b}	271^{ab}	NS	0.06	0.14	0.03	0.03	23.8
EMNS[5] (g of microbial N/kg of degraded substrate OM)	21.0	24.8	25.7	22.1	22.5	26.5	NS	0.02	NS	NS	NS	2.25
Final pH	6.72	6.69	6.70	6.62	6.61	6.62	< 0.01	NS	NS	NS	NS	0.037

[1]C = crotonic acid; Lz = lumazine; [2]SEM = standard error of the mean; [3]PMOM = proportion of substrate and additives OM incorporated into microbial biomass; [4]EMOMS = efficiency of microbial OM synthesis (substrate OM basis); [5]EMNS = efficiency of microbial N synthesis (substrate OM basis); [6]NS = non-significant ($P > 0.15$).

0.6 mM increased ($P < 0.05$) microbial OM, while at 8 mM crotonic acid, microbial OM was higher ($P < 0.05$) at 0.6 than at 0.3 mM lumazine. Crotonic acid tended ($P = 0.09$) to increase production of microbial OM. There were no interactions for the production of microbial N, which was increased both by lumazine ($P < 0.01$) and crotonic acid ($P < 0.05$). There were linear by linear and linear by quadratic ($P = 0.03$) interactions for the efficiency of microbial OM synthesis. At 0 mM crotonic acid, lumazine at 0.3 mM increased ($P < 0.05$) the efficiency of microbial OM synthesis, but there was no effect of lumazine at 8 mM of crotonic acid. There were no interactions for the efficiency of microbial N synthesis, which was increased ($P = 0.02$) by lumazine, and not affected by crotonic acid. No interactions were present for final pH, which was decreased ($P < 0.01$) by crotonic acid, and not affected by lumazine (Table 7-1).

Experiment 2

Approximately one third of 3-butenoic acid disappeared by 24 h of incubation across all lumazine concentrations. 3-Butenoic acid completely disappeared by 48 h of incubation at all lumazine concentrations (Figure 7-2).

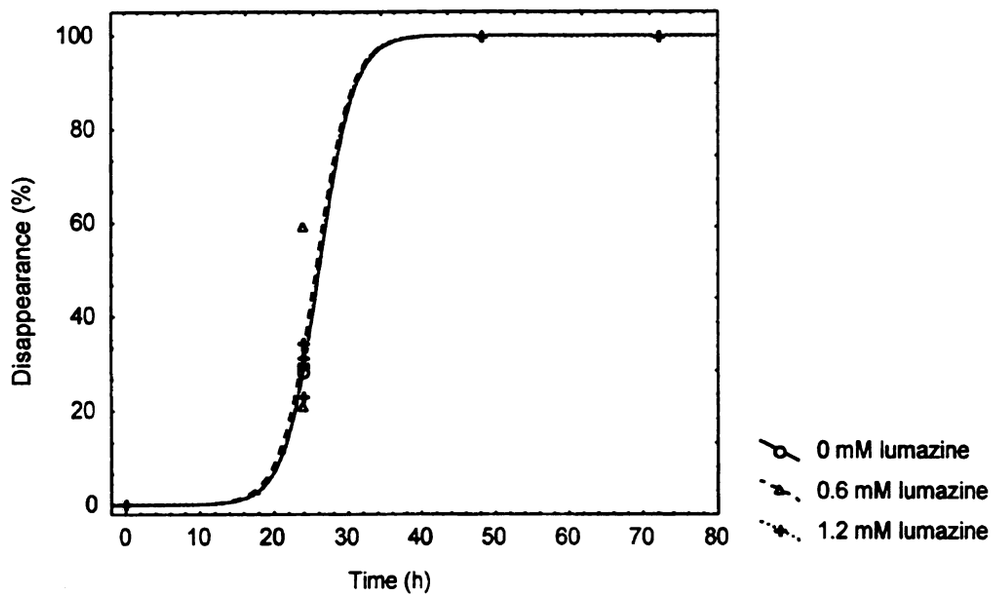

Figure 7-2 3-Butenoic acid disappearance (Experiment 2)

There were no interactions for OM, N and NDF degradation. Lumazine did not affect OM degradation (Table 7-2). At 1.2 mM, lumazine decreased N degradation (P = 0.02; quadratic response). In contrast, 3-butenoic acid increased (P = 0.03) N degradation, although the increase was not enough to compensate for the decrease caused by lumazine at 1.2 mM. Apparent NDF degradation was not influenced by either additive. There were no interactions for the proportion of total OM incorporated into microbial biomass, which tended ($P < 0.10$) to increase with added lumazine. There was a tendency (P = 0.14) for a linear by quadratic interaction for microbial OM production. Lumazine increased the production of microbial OM (P = 0.01). 3-Butenoic acid increased ($P < 0.05$) microbial OM production only at 1.2 mM lumazine. There were no interactions for microbial N production. Lumazine increased ($P < 0.01$; quadratic response) microbial N production, and 3-butenoic acid did not affect it. There were no

Table 7-2. Effects of lumazine and 3-butenoic acid on 72 h in vitro degradation and microbial biomass production (Experiment 2)

3-Butenoic acid (mM)	0			4			Probabilities[1]					SEM[2]
Lumazine (mM)	0	0.6	1.2	0	0.6	1.2	B	Lz	Lz^2	B x Lz	B x L^2	
True OM degradation (%)	44.5	45.4	42.8	45.5	46.1	44.2	NS[6]	NS	NS	NS	NS	1.69
True N degradation (%)	69.5	67.4	60.6	73.6	73.6	62.0	0.03	NS	0.02	NS	NS	2.94
Apparent NDF degradation (%)	41.0	41.8	39.5	40.9	40.0	39.6	NS	NS	NS	NS	NS	1.45
PMOM[3]	0.20	0.22	0.22	0.19	0.20	0.22	NS	0.10	NS	NS	NS	0.017
Microbial OM (mg)	474^{c}	563^{ab}	550^{b}	525^{bc}	570^{ab}	615^{a}	0.08	0.01	< 0.05	NS	0.14	32.5
Microbial N (mg)	45.5	55.3	54.4	47.9	54.5	56.8	NS	< 0.01	0.02	NS	NS	2.89
EMOMS[4] (g of microbial OM /kg of degraded substrate OM)	196	227	235	211	226	255	NS	0.02	NS	NS	NS	18.2
EMNS[5] (g of microbial N/kg of degraded substrate OM)	18.9	22.3	23.2	19.3	21.6	23.5	NS	< 0.01	NS	NS	NS	1.65
Final pH	6.73	6.73	6.74	6.68	6.69	6.71	< 0.01	NS	NS	NS	NS	0.016

[1]B = 3-butenoic acid; Lz = lumazine;.

[2]SEM = standard error of the mean.

[3]PMOM = proportion of substrate and additives OM incorporated into microbial biomass.

[4]EMOMS = efficiency of microbial OM synthesis (substrate OM basis).

[5]EMNS = efficiency of microbial N synthesis (substrate OM basis).

[6]NS = non-significant ($P > 0.15$).

interactions for the efficiencies of synthesis of microbial OM and N, both of which were increased by lumazine and not affected by 3-butenoic acid.

There were no interactions for final pH. 3-Butenoic acid decreased ($P < 0.01$) the final pH, while lumazine had no effect (Table 7-2).

Experiment 3

At 0 mM propynoic acid, all of crotonic acid disappeared by the end of the incubation period, but only 63 and 69% disappeared at 2 and 4 mM propynoic acid, respectively ($P < 0.01$; quadratic response). Disappearance of propynoic acid at the end of the incubation period was lower than 10% for all treatments. There was a tendency ($P = 0.12$) for an interaction for propynoic disappearance. At 4 mM crotonic acid, it was higher ($P < 0.05$) with 2 mM propynoic acid than with 4 mM, while there was no difference at 0 mM crotonic acid.

There were no interactions for OM degradation. Propynoic acid decreased ($P < 0.01$; quadratic response) OM degradation from 37.6 to 25.1%, while crotonic acid had

Table 7-3. Effects of propynoic acid and crotonic acid on 72 h in vitro degradation and microbial biomass production (Experiment 3)

Crotonic acid (mM)	0			4			Probabilities[1]					SEM[2]
Propynoic acid (mM)	0	2	4	0	2	4	C	P	P²	C x P	C x P²	
Crotonic acid disappearance (%)	-	-	-	100	63.2	68.8	-	< 0.01	< 0.01	-	-	2.33
Propynoic acid disappearance (%)	-	1.5^{ab}	1.4^{ab}	-	7.9^{a}	0.8^{b}	NS[4]	0.11	-	0.12	-	1.24
True OM degradation (%)	39.3	27.0	26.0	35.8	28.7	24.2	NS	< 0.01	0.02	NS	NS	3.13
True N degradation (%)	43.7^{a}	20.7^{c}	1.28^{d}	29.2^{b}	23.3^{bc}	1.57^{d}	< 0.01	< 0.01	NS	< 0.01	< 0.01	3.07
Apparent NDF degradation (%)	36.9^{a}	25.6^{c}	25.0^{c}	30.9^{b}	24.3^{c}	25.2^{c}	0.02	< 0.01	< 0.01	0.07	NS	2.77
PMOM[3]	0.32	0.56	0.62	0.36	0.52	0.60	NS	< 0.01	0.09	NS	NS	0.071
Microbial OM (mg)	695	824	873	805	913	868	0.03	< 0.01	NS	NS	NS	69.3
Microbial N (mg)	63.7^{c}	83.2^{a}	82.1^{a}	70.7^{b}	86.6^{a}	83.6^{a}	< 0.01	< 0.01	< 0.01	0.10	NS	2.80
EMOMS[4] (g of microbial OM /kg of degraded substrate OM)	323	559	621	410	583	688	NS	< 0.01	NS	NS	NS	92.7
EMOMT[6] (g of microbial OM /kg of total degraded OM)	323	559	620	361	524	596	NS	< 0.01	0.09	NS	NS	71.3
EMNS[5] (g of microbial N/kg of degraded substrate OM)	29.7	56.6	58.3	36.2	55.5	66.4	NS	< 0.01	0.08	NS	NS	8.70
EMNT[7] (g of microbial N/kg of total degraded OM)	29.7	56.5	58.2	31.8	49.8	57.5	NS	< 0.01	0.02	NS	NS	6.93
Final pH	6.70^{a}	6.72^{a}	6.71^{a}	6.65^{b}	6.71^{a}	6.62^{b}	0.02	NS	NS	< 0.01	< 0.01	0.028

[1]C = crotonic acid; P = propynoic acid; [2]SEM = standard error of the mean; [3]PMOM = proportion of substrate and additives OM incorporated into microbial biomass; [4]NS = non-significant ($P > 0.15$).

no effect (Table 7-3). There were linear by linear and linear by quadratic interactions ($P < 0.01$) on N degradation. Propynoic acid decreased ($P < 0.01$) N degradation to almost zero. Crotonic acid decreased ($P < 0.05$) N degradation only at 0 mM propynoic acid. There was a tendency ($P = 0.07$) for a linear by linear interaction for NDF degradation. Propynoic acid decreased ($P < 0.01$; quadratic response) NDF degradation. Crotonic acid decreased ($P < 0.05$) NDF degradation only at 0 mM propynoic acid. There were no interactions for the proportion of OM incorporated into microbial biomass. Propynoic acid increased ($P < 0.01$; quadratic response) the proportion of OM incorporated into microbial biomass, and crotonic acid had no effect. There were no interactions for microbial OM production, which was increased by both additives. There was a tendency ($P = 0.10$) for a linear by linear interaction for microbial N production. Propynoic acid increased ($P < 0.01$; quadratic response) microbial N production. Crotonic acid increased ($P < 0.05$) microbial N production at 0 mM propynoic acid. There were no interactions for microbial OM and N synthetic efficiencies. Propynoic acid improved ($P < 0.01$) microbial OM and N synthetic efficiencies, while crotonic acid had no effect.

There were linear and linear by quadratic interactions ($P < 0.01$; Table 7-3) on final pH. Propynoic acid did not affect final pH at 0 mM crotonic acid, but at 4 mM crotonic acid pH was minimum ($P < 0.05$) at 2 mM propynoic acid.

Experiment 4

Less than 20% propynoic acid disappeared by the end of the incubation period (Table 7-4). Significant effects and interactions were of little biological importance.

Table 7-4. Effects of propynoic acid and 3-butenoic acid on 72 h in vitro degradation and microbial biomass production (Experiment 4)

3-Butenoic acid (mM)	0			4			Probabilities[1]					SEM[2]
Propynoic acid (mM)	0	2	4	0	2	4	B	P	P^2	B x P	B x P^2	
3-Butenoic acid disappearance (%)	-	-	-	100	100	99.5	-	NS[4]	NS	-	-	0.24
Propynoic acid disappearance (%)	-	16.6^{b}	19.8^{a}	-	19.5^{a}	19.2^{a}	0.04	0.02	-	< 0.01	-	0.28
True OM degradation (%)	31.9^{a}	24.0^{c}	25.7^{bc}	30.9^{ab}	29.9^{ab}	27.1^{abc}	NS	0.01	0.04	0.06	0.08	2.94
True N degradation (%)	44.6^{a}	39.9^{ab}	32.5^{b}	40.8^{ab}	48.9^{a}	32.6^{b}	NS	0.04	NS	0.02	0.02	6.45
Apparent NDF degradation (%)	26.3^{a}	15.4^{c}	17.2^{bc}	26.6^{a}	21.1^{b}	19.0^{bc}	NS	< 0.01	< 0.01	0.10	0.11	2.71
PMOM[3]	0.34^{c}	0.57^{a}	0.51^{ab}	0.33^{c}	0.43^{b}	0.47^{b}	NS	< 0.01	< 0.01	0.03	0.03	0.047
Microbial OM (mg)	596^{c}	754^{b}	725^{b}	642^{c}	822^{a}	837^{a}	0.11	< 0.01	< 0.01	0.11	NS	33.1
Microbial N (mg)	53.3	73.0	70.2	58.3	77.8	76.7	0.02	< 0.01	< 0.01	NS	NS	2.46
EMOMS (g of microbial OM /kg of degraded substrate OM)	343^{b}	576^{a}	521^{a}	391^{b}	505^{a}	567^{a}	NS	< 0.01	< 0.01	0.07	0.06	57.2
EMOMT (g of microbial OM /kg of total degraded OM)	343^{c}	568^{a}	505^{ab}	329^{c}	428^{b}	468^{b}	NS	< 0.01	< 0.01	0.03	0.03	47.4
EMNS (g of microbial N/kg of degraded substrate OM)	30.6^{b}	55.8^{a}	50.5^{a}	35.4^{b}	47.7^{a}	52.0^{a}	NS	< 0.01	< 0.01	0.05	0.06	5.30
EMNT (g of microbial N/kg of total degraded OM)	30.6^{d}	55.0^{a}	48.9^{ab}	30.4^{d}	40.5^{c}	42.9^{bc}	NS	< 0.01	< 0.01	0.01	0.02	4.33
Final pH	6.84	6.82	6.79	6.77	6.75	6.74	0.09	NS	NS	NS	NS	0.056

[1]B = 3-butenoic acid; P = propynoic acid; [2]SEM = standard error of the mean; [3]PMOM = proportion of substrate and additives OM incorporated into microbial biomass; [4]NS = non-significant ($P > 0.15$).

There were tendencies for linear ($P = 0.06$) and linear by quadratic ($P = 0.08$) interactions for OM degradation. Propynoic acid decreased ($P < 0.05$) OM degradation at 0, but not at 4 mM 3-butenoic acid (Table 7-4). 3-Butenoic acid increased ($P < 0.05$) OM degradation only at 2 mM propynoic acid. Propynoic acid decreased ($P = 0.04$) N degradation, while 3-butenoic acid did not affect it. There were tendencies for linear by linear ($P = 0.10$) and linear by quadratic ($P = 0.11$) interactions for NDF degradation. Propynoic acid decreased ($P < 0.01$) NDF degradation. 3-Butenoic acid increased ($P < 0.05$) NDF degradation only at 2 mM propynoic acid. There were linear by linear and linear by quadratic interactions ($P = 0.03$) for the proportion of OM incorporated into microbial biomass. Propynoic acid increased ($P < 0.01$; quadratic response) the proportion of total OM incorporated into microbial biomass. At 2 mM propynoic acid, 3-butenoic acid lowered ($P < 0.05$) , the proportion of OM incorporated into microbial biomass. There was a tendency ($P = 0.11$) for an interaction for microbial OM production. Propynoic acid increased ($P < 0.01$; quadratic response) microbial OM production. Butenoic acid increased ($P < 0.05$) microbial OM production at 2 and 4 mM propynoic acid. There were no interactions for microbial N production, which was increased both by propynoic acid ($P < 0.01$; quadratic response) and 3-butenoic acid ($P = 0.02$). Propynoic acid increased ($P < 0.01$; quadratic response) the efficiency of microbial OM synthesis calculated on a degraded substrate basis, while 3-butenoic acid did not affect it. There were linear by linear and linear by quadratic interactions ($P = 0.03$) for the efficiency of microbial OM synthesis calculated on a total degraded OM basis. Propynoic acid increased ($P < 0.01$; quadratic response) the efficiency of microbial OM synthesis calculated on a total degraded OM basis, while 3-butenoic acid

decreased ($P < 0.05$) it only at 2 mM propynoic acid. Propynoic acid increased ($P < 0.01$; quadratic response) the microbial efficiency of N synthesis calculated on a degraded substrate basis, while 3-butenoic acid did not affect it. There were linear by linear ($P = 0.01$) and linear by quadratic ($P = 0.02$) interactions for the microbial efficiency of N synthesis calculated on a total degraded OM basis. Propynoic acid increased ($P < 0.01$; quadratic response) the microbial efficiency of N synthesis calculated on a total degraded OM basis, while 3-butenoic acid decreased ($P < 0.05$) it only at 2 mM propynoic acid.

There were no interactions for final pH. 3-Butenoic acid tended ($P = 0.09$) to decrease final pH, while propynoic acid did not affect it (Table 7-4).

Experiment 5

More than 97% of ethyl 2-butynoate disappeared after 24 h of incubation. There were no interactions for ethyl 2-butynoate disappearance. Disappearance was not affected by its initial concentration or by the presence of crotonic acid (Table 7-5). Virtually all crotonic acid disappeared after 48 h of incubation (Figure 7-3). However, disappearance during the first 24 h was greater ($P < 0.01$) at 0 mM ethyl 2-butynoate.

There were no interactions for OM, N and NDF degradation. Ethyl 2-butynoate at 8 mM decreased OM degradation from 36.0 to 30.6%. However, ethyl 2-butynoate at 4 mM did not impair OM degradation ($P = 0.04$; quadratic response; Table 7-5). Crotonic acid did not affect OM degradation. Ethyl 2-butynoate strongly decreased ($P < 0.05$; quadratic response) N degradation from 47.9 to 19.5%, while there was no effect of crotonic acid. The effect of ethyl 2-butynoate on apparent NDF degradation was

Table 7-5. Effects of ethyl 2-butynoate and crotonic acid on 72 h in vitro degradation and microbial biomass production (Experiment 5)

Crotonic acid (mM)	0			4			Probabilities[1]					SEM[2]
Ethyl 2-butynoate (mM)	0	4	8	0	4	8	C	EB	EB[2]	C x EB	C x EB[2]	
Ethyl 2-butynoate disappearance (%)[3]	-	98.7	97.5	-	97.2	97.3	NS[5]	NS	-	NS	-	0.58
True OM degradation (%)	34.5	37.6	29.6	37.4	36.8	31.6	NS	NS	0.04	NS	NS	3.49
True N degradation (%)	47.2	42.1	21.4	48.6	37.6	17.6	NS	NS	< 0.05	NS	NS	5.61
Apparent NDF degradation (%)	30.8	37.6	29.6	35.5	38.2	31.4	0.11	< 0.01	< 0.01	NS	NS	2.98
PMOM[4]	0.34	0.29	0.35	0.26	0.28	0.28	NS	NS	NS	NS	NS	0.056
Microbial OM (mg)	625	678	794	604	731	743	NS	0.02	NS	NS	NS	76.6
Microbial N (mg)	58.0	68.5	80.7	58.3	72.3	76.2	NS	< 0.01	NS	NS	NS	7.27
EMOMS (g of microbial OM /kg of degraded substrate OM)	340	335	502	296	364	433	NS	0.02	NS	NS	NS	77.2
EMOMT (g of microbial OM /kg of total degraded OM)	340	287	351	262	281	280	NS	NS	NS	NS	NS	56.1
EMNS (g of microbial N/kg of degraded substrate OM)	31.7	34.0	50.8	28.6	36.0	44.4	NS	< 0.01	NS	NS	NS	7.62
EMNT (g of microbial N/kg of total degraded OM)	31.7	29.1	35.6	25.3	27.7	28.7	NS	NS	NS	NS	NS	5.61
Final pH	6.79	6.71	6.70	6.74	6.70	6.64	NS	< 0.01	NS	NS	NS	0.032

[1]C = crotonic acid; EB = ethyl 2-butynoate.

[2]SEM = standard error of the mean.

[3]by 24 h.

[4]PMOM = proportion of substrate and additives OM incorporated into microbial biomass.

[5]NS = non-significant ($P > 0.15$).

quadratic, with a maximum at 4 mM. Crotonic acid tended ($P = 0.11$) to increase apparent NDF degradation. There were no interactions for the proportion of OM incorporated into microbial biomass, which was not affected by either additive. There were no interactions for microbial OM and N production and the synthetic efficiencies. Despite the decrease in OM degradation, ethyl 2-butynoate increased the production of microbial OM ($P = 0.02$) and N ($P < 0.01$) through increased efficiencies of synthesis. However, there was no improvement in the efficiencies when total OM disappearance was accounted. Crotonic acid had no effect on the production of microbial OM and N, or their efficiencies of synthesis.

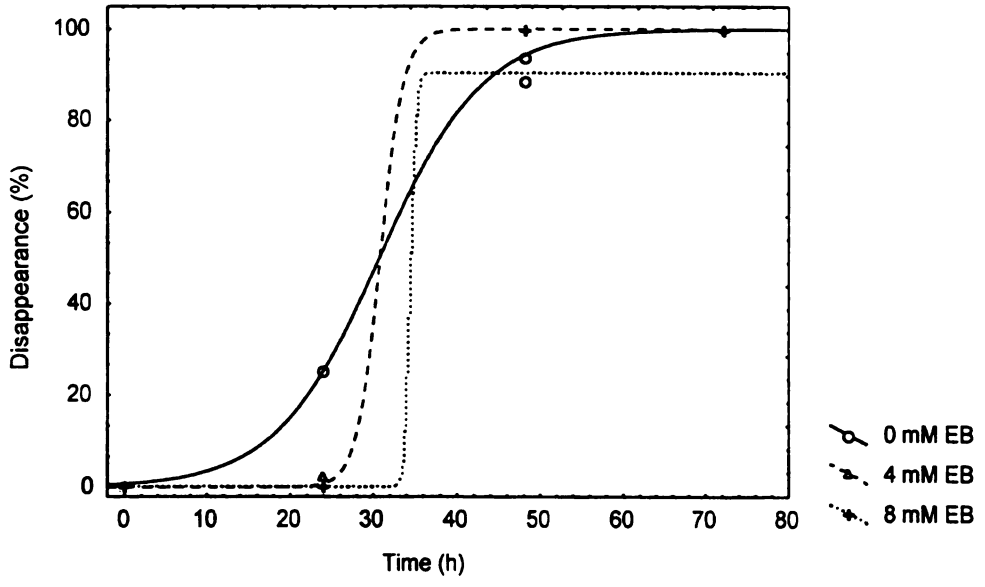

Figure 7-3 Crotonic acid disappearance (Experiment 5)

There were no interactions for final pH. Ethyl 2-butynoate decreased ($P < 0.01$) final pH, while crotonic acid did not affect it (Table 7-5).

Experiment 6

More than 95% of ethyl 2-butynoate disappeared after 24 h of incubation. There were no interactions for ethyl 2-butynoate disappearance. Disappearance was not affected by its initial concentration or by the presence of 3-butenoic acid (Table 7-6). All of 3-butenoic acid had disappeared by 48 h of incubation. However, the disappearance of 3-butenoic acid's was considerably smaller at 24 h incubation, especially at 8 mM ethyl 2-butynoate (Figure 7-4).

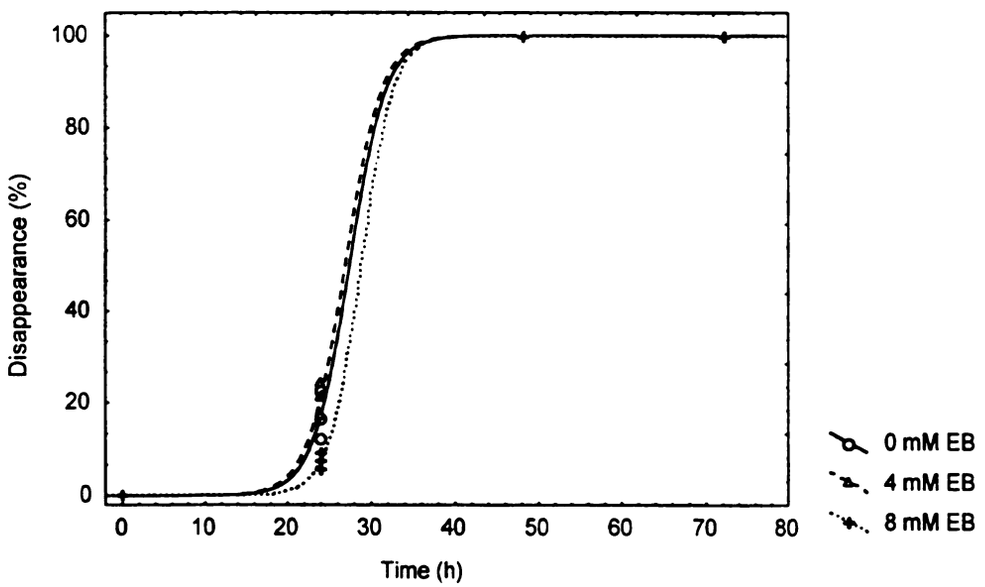

Figure 7-4 3-Butenoic acid disappearance (Experiment 6)

There were no interactions for OM or N degradation. Ethyl 2-butynoate did not affect OM degradation, but N degradation was decreased ($P < 0.01$) from 71.0 to 56.6% (Table 7-6). There was no effect of 3-butenoic acid upon OM or N degradation. NDF degradation and the proportion of disappeared OM incorporated into microbial biomass were similar among treatments. Tendencies to increase the efficiencies of microbial OM ($P = 0.08$) and N ($P = 0.06$)

Table 7-6. Effects of ethyl 2-butynoate and 3-butenoic acid on 72 h in vitro degradation and microbial biomass production (Experiment 6)

3-Butenoic acid (mM)	0			4			Probabilities[1]					SEM[2]
Ethyl 2-butynoate (mM)	0	4	8	0	4	8	B	EB	EB[2]	B x EB	B x EB[2]	
Ethyl 2-butynoate disappearance (%)[3]	-	97.1	96.9	-	97.0	95.5	NS[5]	NS	-	NS	-	0.43
True OM degradation (%)	43.9	44.8	38.0	35.9	45.7	39.8	NS	NS	NS	NS	NS	5.86
True N degradation (%)	72.3	67.4	57.7	69.8	69.4	55.5	NS	< 0.01	NS	NS	NS	5.20
Apparent NDF degradation (%)	39.5[a]	39.5[a]	31.7[a]	30.5[a]	40.0[a]	34.9[a]	0.12	NS	0.10	0.12	NS	6.30
PMOM[4]	0.20[a]	0.26[a]	0.26[a]	0.30[a]	0.24[a]	0.21[a]	NS	NS	NS	0.11	NS	0.073
Microbial OM (mg)	485[c]	712[a]	723[a]	567[bc]	730[a]	666[ab]	0.09	< 0.01	< 0.01	0.07	NS	61.8
Microbial N (mg)	45.2	71.4	69.4	50.8	70.9	67.6	NS	< 0.01	< 0.01	NS	NS	5.45
EMOMS (g of microbial OM / kg of degraded substrate OM)	202[a]	291[a]	349[a]	352[a]	293[a]	306[a]	0.08	0.08	NS	0.10	NS	95.5
EMOMT (g of microbial OM / kg of total degraded OM)	202[a]	256[a]	263[a]	297[a]	236[a]	214[a]	NS	NS	NS	0.11	NS	72.9
EMNS (g of microbial N/ kg of degraded substrate OM)	18.8	29.2	33.5	31.6	28.5	31.1	0.12	0.06	NS	NS	NS	8.65
EMNT (g of microbial N/ kg of total degraded OM)	18.8	25.7	25.2	26.6	22.9	21.7	NS	NS	NS	NS	NS	6.63
Final pH	6.76	6.75	6.69	6.71	6.75	6.63	NS	0.12	NS	NS	NS	0.047

[1]B = 3-Butenoic acid; EB = ethyl 2-butynoate.

[2]SEM = standard error of the mean.

[3]by 24 h.

[4]PMOM = proportion of substrate and additives OM incorporated into microbial biomass.

[5]NS = non-significant ($P > 0.15$).

synthesis calculated on a degraded substrate OM basis allowed ethyl 2-butynoate to increase ($P < 0.01$; quadratic response) the production of microbial OM and N production. However, there was no improvement in synthetic efficiencies when total OM was accounted. 3-Butenoic acid did not affect microbial OM and N production or their efficiencies of synthesis.

There were no interactions for final pH. Ethyl 2-butynoate tended ($P = 0.12$) to decrease the final pH, while 3-butenoic acid had no effect (Table 7-6).

Discussion

Lumazine, propynoic acid, and ethyl 2-butynoate decreased N degradation. However, their negative effects on OM and NDF degradation were milder or non-existent. As NDF was the major non-nitrogenous component of the substrate OM, the effects of the inhibitors on OM and NDF degradation were similar. Lumazine did not affect OM or NDF degradation. In Experiment 5, ethyl 2-butynoate at 8 mM, but not at 4 mM, decreased OM and NDF degradation. In Experiment 6, ethyl 2-butynoate did not decrease OM or NDF degradation at any concentration. Propynoic acid had the most negative effects on OM and NDF degradation. However, in Experiment 3, the inhibition exhorted by propynoic on OM and NDF degradation was lower than the inhibition of N degradation.

It is unlikely that the decreased OM, N, or NDF degradation, could have been caused by low pH. Although ethyl 2-butynoate and some propynoic acid combinations decreased final pH, pH was not sufficiently reduced to impair degradation (Ørskov and Ryle, 1990).

As the additives affected more proteolysis than fibrolysis, it is possible that the additives were more toxic to proteolytic than to fibrolytic microorganisms. Proteolytic activity is quite widespread among ruminal bacteria, although the main cellulolytic bacteria are possible exceptions (Wallace et al., 1997). Among cell-free extracts from 14 different entodiniomorphid protozoa, the cellulolytic species had the lowest proteolytic activity (Coleman, 1983). Reports on the proteolytic activity of ruminal fibrolytic fungi are somewhat conflicting, although their role in proteolyisis in vivo is probably minor (Wallace et al., 1997). Thus, there seems to be little overlapping between ruminal proteolytic and cellulolytic microbial species, and it is possible that the inhibitors of methanogenesis herein studied were more toxic to the former than the latter.

However, the fact that the three different compounds affected proteolysis more than fibrolysis suggests that a more general explanation related to the inhibition of CH_4 production could be possible. Propynoic acid and ethyl 2-butynoate increased the accumulation of H_2. It has been observed that H_2 accumulation resulting from the inhibition of CH_4 formation decreased the redox potential of ruminal cultures (Sauer and Teather, 1987). An increase in H_2 pressure interferes with the interspecies H transfer and the re-oxidation of cofactors (Nagaraja et al., 1997). Deamination releases one mole of reducing equivalent pairs per mole of NH_4^+ released (Voet and Voet, 1995). When hydrogenase activity and subsequent CH_4 formation were inhibited by CO, the catabolism of amino acids was decreased. Deamination of branched-chain amino acids, which are highly reduced, was particularly inhibited (Russell and Martin, 1984). Furthermore, deamination of branched-chain amino acids was inhibited by NADH (Hino

and Russell, 1985). However, these results were not entirely reproducible with another inhibitor of methanogenesis, chloroform (Russell and Martin, 1984).

Possibly, the inhibition of CH_4 production had adverse consequences on deamination, which could result in decreased proteolytic activity if amino acids accumulated (Blackburn, 1968; Cotta and Hespell, 1986), and/or NH_4^+ concentration decreased (Blackburn, 1968). Other work, however, did not find marked alterations in proteolytic activity of ruminal bacteria caused by changes in the concentrations of NH_4^+, amino acids or peptides (Blackburn, 1968), and it was concluded that proteases are constitutive (Wallace and Brammall, 1985).

An inhibition of deamination is consistent with the decreased NH_4^+ concentration observed with propynoic acid, ethyl 2-butynoate, and lumazine. Yet, responses in VFA products of amino acid catabolism have not always been consistent with a decrease in deamination (Chapters 3, 4, and 6). Lumazine decreased isovalerate, but caused large increases in isobutyrate production. Isovalerate and isobutyrate are products of leucine and valine catabolism, respectively (Nagaraja et al., 1997). Propynoic acid has been shown to decrease the production of isobutyrate, valerate, and isovalerate, although, strangely, the responses were sometimes quadratic. Valerate is a product of proline catabolism (Nagaraja et al., 1997). Ethyl 2-butynoate decreased valerate and isovalerate, but greatly increased isobutyrate production.

Another problem with this explanation for greater reduction in proteolysis than fibrolysis, is that hexose fermentation, the same as deamination, releases metabolic H. Glycolysis releases two moles of reducing equivalent pairs per mole of glucose fermented (Voet and Voet, 1995), and more metabolic H is released when acetate is the end product

of fermentation. Then, a decrease in redox potential should inhibit both proteolysis and cellulolysis.

Proteolytic activity can also be decreased by reducing agents as a result of hydrogenation of cysteine residues involved in the catalytic mechanism (Wallace and Brammall, 1985). Sodium sulfide, L-cysteine and dithiothreitol decreased proteolytic activity by *Butyrivibrio fibrisolvens* (Cotta and Hespell, 1986). Dithiothreitol also decreased the proteolytic activity of both mixed ruminal fluid and monocultures of ruminal bacterial species (Wallace and Brammall, 1985). However, other results in which proteolytic activity of *Prevotella* (*Bacteroides*) *ruminicola* was increased by dithiothreitol and cysteine have also been reported (Hazlewood et al., 1981).

Under most situations, an inhibition of ruminal proteolysis is not undesirable. Protein fermentation in the rumen is generally considered wasteful (Russell, 1983). The conversion of feed protein into microbial protein requires energy, part of dietary amino acids are fermented, and NH_4^+ released is only partially captured into microbial protein (Nagaraja et al., 1997), increasing N voided in the environment.

Crotonic acid was ineffective in improving OM, N, or NDF degradation. There was some improvement in N degradation by supplementing lumazine at 0.6 mM with 3-butenoic acid, but no consistent improvements in degradation of OM, N or NDF were observed with 3-butenoic acid. Previous results had shown that these additives could improve apparent OM fermentation (Chapter 3). A shift in degraded OM towards more end more end products of fermentation and less microbial biomass may explain the increase in apparent OM degradation with no change in true degradation. However, only the combination of crotonic acid with lumazine at 0.3 mM, and 3-butenoic acid with

propynoic acid at 2 mM, decreased the proportion of disappeared OM incorporated into microbial biomass. Overall, crotonic acid only tended ($P = 0.11$) to decrease the proportion of total OM (substrate plus additives) incorporated into microbial biomass in Experiment 1, and did not affect it in Experiments 3 and 5. 3-Butenoic acid did not affect the proportion of OM incorporated into microbial biomass in any of the experiments. It is not clear therefore why crotonic acid and 3-butenoic acid stimulated apparent OM degradation in previous experiments, but failed to improve true OM degradation in the present experiments.

Nitrogen degradation in the absence of added additives ranged between 43.7 and 72.3% across experiments. The reasons for this ample variation are not apparent, as the same source of ground hay was used in all the experiments. Differences in the proteolytic activity of the ruminal fluid used as inoculum are unlikely as the incubations were conducted for 72 h.

Unexpectedly, all of the inhibitors of CH_4 formation improved the efficiencies of production of microbial OM and N, expressed on a truly degraded substrate OM basis. Previous reports of the effects of the inhibition of ruminal CH_4 production on microbial efficiency of N synthesis are scarce. No changes in the efficiency of microbial N synthesis were found when CH_4 production was inhibited with 2-trichloromethyl-4-dichloromethylene benzo[1,3] dioxin 6-carboxylic acid or monensin (Stanier and Davies, 1981). Unfortunately, the efficiency of microbial N synthesis was expressed on an apparent OM degradation basis in that study.

It is tempting to think that the improvements in microbial efficiency were a general consequence of changes in H dynamics. The inhibition of CH_4 formation

decreases the redox potential (Sauer and Teather, 1987), which could favor anabolic processes that incorporate reducing equivalents, like amination and fatty acid synthesis (Voet and Voet, 1995).

However, ethyl 2-butynoate did not improve the efficiencies of microbial OM and N synthesis when the disappearance of OM from the additives was considered. It is apparent therefore that ethyl 2-butynoate was used as a source of C and/or energy. Figure 7-5 shows that disappearance of C from ethyl 2-butynoate would be sufficient to explain the increase in microbial C observed. In contrast, the potential supply of C from disappeared propynoic acid would be largely insufficient to explain the increases observed in microbial C.

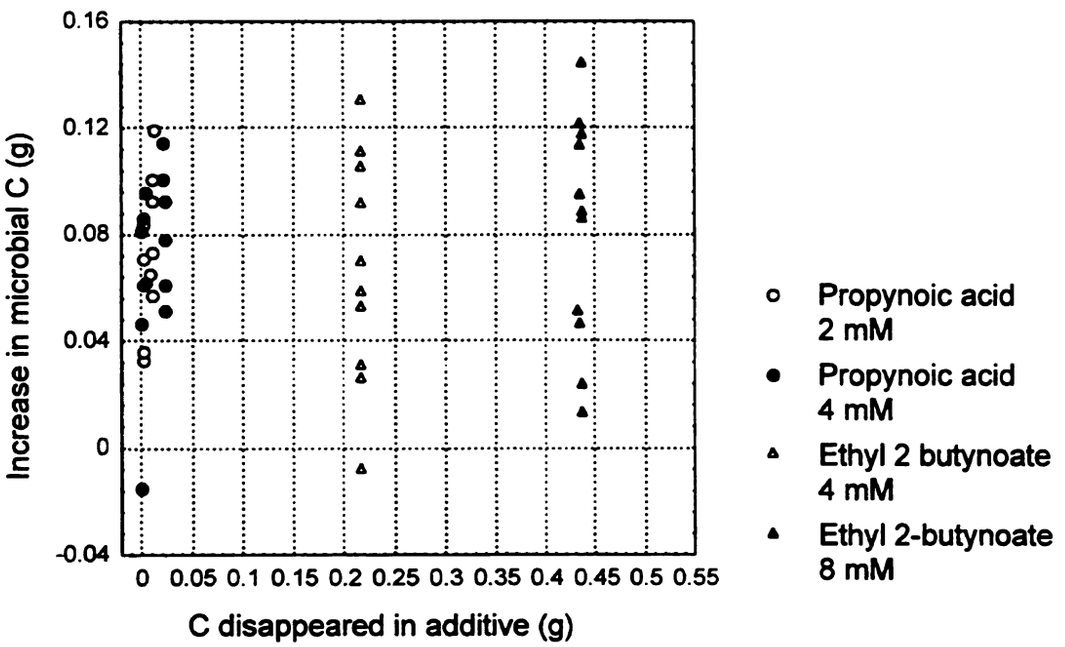

Figure 7-5 Relationship between C disappeared from additives and the increase in microbial C synthesis

Propynoic acid increased microbial efficiency of OM and N synthesis without being used as a C or energy source. It also increased the proportion of OM that was incorporated into microbial biomass. Protozoa release a significant proportion of digested bacterial material into the medium and increase VFA concentration (Williams and Coleman, 1997). Propynoic acid has been shown to decrease VFA concentration (Chapters 3 and 6). It is then possible that the shift in the partition of disappeared OM towards microbial biomass and less fermentation products was caused by antiprotozoal activity of propynoic acid. This would decrease bacterial predation and N turnover, and increase the production of microbial N (Williams and Coleman, 1997).

An improvement in the efficiency of microbial N synthesis may also occur if propynoic acid inhibited deamination more than proteolysis, as this would increase the availability of amino acids. There is evidence that amino acids and peptides can stimulate the efficiency of microbial N synthesis (Leng and Nolan, 1984), although this is generally observed with rapidly degradable energy sources. It may not be true for energy substrates that are degraded more slowly such as hay used in the present study (Wallace et al., 1997).

A lumazine derivative, 6, 7-dimethyl-8-ribityllumazine, is a riboflavine precursor (Weimar and Neims, 1975), and therefore an FAD precursor (Voet and Voet, 1995). If lumazine is used to synthesize FAD, this may relieve some of the decrease in redox potential that occurs when CH_4 production is inhibited (Sauer and Teather, 1987). It could be of interest to evaluate whether there are responses in degradation and microbial biomass synthesis to riboflavine when methanogenesis is inhibited.

Conclusions

All the inhibitors of methanogenesis decreased N degradation. Organic matter and NDF degradation were decreased by propynoic acid and ethyl 2-butynoate at 8 mM. Possible reasons for a greater inhibition of N than OM and NDF degradation are: 1) a greater toxicity to proteolytic than cellulolytic microorganisms (as NDF comprised most of non-nitrogenous OM); 2) inhibition of proteolysis due to less deamination resulting from a lower redox potential; and/or 3) inhibition of proteolysis as a result of hydrogenation of cysteine residues in catalytic sites. Neither crotonic acid nor 3-butenoic acid were shown to consistently improve OM, N, or NDF degradation, and it does not appear to be beneficial to use these acids to relieve the depression in degradation caused by inhibitors of CH_4 formation.

Decreases in CH_4 production caused by lumazine have been erratic, and lower than with other additives. However, lumazine did not impair OM or NDF degradation, and it increased microbial N and OM production. It decreased substrate proteolysis, which can decrease N flow into NH_4^+ in the rumen and reduce N release into the environment.

Ethyl 2-butynoate at a concentration of 4 mM did not impair OM or NDF degradation. Methane production has been shown to decrease by approximately 50% at 4 mM ethyl 2-butynoate (Chapter 6), although 6 mM caused only a 24% decrease (Chapter 3). The additive was totally fermented and increased microbial N production. It could decrease N flow to NH_4^+ in the rumen, improve N retention by the animal, and decrease N voided to the environment. Ethyl 2-butynoate was catabolized within 24 h of

fermentation, which would decrease the risks of toxicity for the animal or the environment.

All three additives improved microbial synthetic efficiencies. Relocation of electrons spared from CH_4 formation may have favored anabolic processes such as amino acids or fatty acid biosynthesis. Alternatively, ethyl 2-butynoate could have improved synthetic efficiencies by being a C source. However, the increases in microbial C caused by propynoic acid were greater than the C disappeared from this additive. The shift in the partition of OM towards more microbial biomass and less fermentation products could indicate a reduction in bacterial lysis. Changes in protozoal numbers could be useful to understand the effects of propynoic acid on degradation and synthetic efficiencies.

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CHAPTER 8

Effects of several inhibitors on pure cultures of ruminal methanogens

Abstract

The effects of five inhibitors of methanogenesis, 2-bromoethanesulfonate (BES), 3-bromopropanesulfonate (BPS), lumazine, propynoic acid, and ethyl 2-butynoate, on CH_4 production of ruminal methanogens *Methanobrevibacter ruminantium*, *Methanosarcina mazei*, and *Methanomicrobium mobile* were examined. *M. ruminantium* was the most sensitive species to BES, propynoic acid, and ethyl 2-butynoate. *M. mazei* was the least sensitive species to those chemical additives, and *M. mobile* was intermediate. Inhibition caused by propynoic acid and ethyl 2-butynoate appeared to be non-competitive. BPS failed to inhibit any of the methanogens. All three species were almost completely inhibited by 50- and 100%-lumazine saturated media, but the inhibition was somewhat lower with a 25%-lumazine saturated media. There were important differences among species of methanogens regarding their sensitivity to the different inhibitors. The presence of resistant species of methanogens should be considered when designing strategies of inhibition of ruminal methanogenesis. Long term changes in the populations of ruminal methanogens caused by inhibitors of methanogenesis need to be investigated.

Introduction

Methane formation in the rumen implies an energy loss for ruminants, and also contributes to global warming. There is, therefore, an interest in decreasing CH_4

production in the rumen (Moss, 1993). Chemical additives with different modes of action have been used to decrease ruminal methanogenesis. Some compounds have been shown to considerably decrease CH_4 production of mixed ruminal cultures (Van Nevel and Demeyer, 1996; Nagaraja et al., 1997).

Although the main interest when evaluating potential inhibitors of methanogenesis is their effects on the mixed ruminal microbial community, it is important to know how individual species of methanogens are affected. If some species are resistant to an inhibitor, its long term use in vivo could result in their selection, and CH_4 formation could return to the levels observed before the inhibitor was fed. For example, 2-bromoethanesulfonate (BES) is a very potent inhibitor of methanogenesis in batch cultures, but ruminal microbiota of sheep adapted to the compound after BES was administered to the animal for three days (Van Nevel and Demeyer, 1996). Also, the relative proportion of methanogenic species can be influenced by ruminant species (Lin et al., 1997), diet composition and management (Rowe et al., 1979; Jarvis et al., 2000), and geographical area (Vicini et al., 1987). This may result in different responses to chemical additives used to inhibit CH_4 production in the rumen. BES (Nagaraja et al., 1997), 3-bromopropanesulfonate (BPS), lumazine, propynoic acid, and ethyl 2-butynoate have been previously studied regarding their effects on CH_4 production by mixed ruminal cultures (see Chapters 3 and 4). The objectives of the present study were to investigate the effects of these five chemicals on CH_4 production of pure cultures of three ruminal methanogens. It was hypothesized that there would be differences among methanogens in their sensitivity to the inhibitors.

Materials and Methods

Cultures

The experiment was conducted at the Department of Biology, Portland State University, Portland, OR, USA. *Methanobrevibacter ruminantium* M1 (OCM 146) and *Methanosarcina mazeii* CW3A (OCM 20) were obtained from the Oregon Collection of Methanogens (Department of Biology, Portland State University, Portland, OR 97207, USA). *Methanomicrobium mobile* 1539 (DSM 1539) was obtained from DSMZ (DSMZ Versand, 38124 Braunschweig, Germany). All three strains have been isolated from ruminal fluid.

Medium preparation

All three cultures were grown in MS medium (Boone et al., 1989), which contained per liter: 4.0 g NaOH, 2.0 g yeast extract (Difco Laboratories, Detroit, MI), 2.0 g Trypticase peptones (BBL Microbiology Systems, Cockeysville, MD), 1.0 g NH_4Cl, 1.0 g $MgCl_2 \cdot 6H_2O$, 0.2 g 2-mercaptoethanesulfonic acid (coenzyme M), 0.4 g $K_2HPO_4 \cdot 3H_2O$, 0.4 g $CaCl_2 \cdot 2H_2O$, 5.0 mg disodium EDTA$\cdot 2H_2O$, 1.5 mg $CoCl_2 \cdot 6H_2O$, 1.0 mg resazurin, 1.0 mg of $MnCl_2 \cdot 4H_2O$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 1.0 mg $ZnCl_2$, 0.4 mg $AlCl_3 \cdot 6H_2O$, 0.3 mg $Na_2WO_4 \cdot 2H_2O$, 0.2 mg $CuCl_2 \cdot 2H_2O$, 0.2 mg $NiSO_4 \cdot 6H_2O$, 0.1 mg H_2SeO_3, 0.1 mg of H_3BO_3, and 0.1 mg $NaMoO_4 \cdot 2H_2O$, 250 mg $Na_2S \cdot 9H_2O$, and 250 mg L-cysteine . Twenty five percent (v/v) of deionized water was replaced with clarified ruminal fluid (Leadbetter and Breznak, 1996). Methanol (30 mM final concentration) was also included in the medium for *M. mazei* (Pol and Demeyer, 1988). Medium was anaerobically mixed and delivered under a 50:50 O_2-free N_2/CO_2 gas mixture. pH of the

medium before delivery into tubes was 6.90. Five milliliters of medium were delivered into 25 mL-Hungate tubes and hermetically sealed with butyl rubber septum and aluminum seals (Bellco Glass Company, Vineland, NJ). Tubes were then autoclaved at 121 °C for 20 min. Approximately 24 h before inoculation, 0.05 mL of a previously autoclaved, O_2-free, 2.5% (w/v) $Na_2S \cdot H_2O$ stock solution, were aseptically added to the tubes through the butyl rubber septum.

Chemicals

Chemical inhibitors studied were BES and BPS at 0, 10, 50, and 250 μM, propynoic acid at 0, 2 and 4 mM, ethyl 2-butynoate at 0, 4 and 8 mM, and lumazine as a 0, 25, 50 or 100% lumazine-saturated medium. BES [Na salt, Sigma B 9008], BPS [Na salt, B2912], and propynoic acid [free acid, Acros 13150-0100] were dissolved in deionized water, filter-sterilized, and 0.1 mL of each solution delivered into the corresponding tubes. Ethyl 2-butynoate [Aldrich 4341-76-8] was directly injected into the tubes through the butyl rubber septum using a 10 μL Hamilton syringe (2.35 and 4.7 μL of ethyl 2-butynoate to achieve 4 and 8 mM, respectively). Two milligrams of lumazine [~12.2 μmoles; Sigma L 0380] were added to 50 mL of medium in a Wheaton bottle, shaken, and left overnight. The presence of visible yellowish particles of lumazine at the bottom of the bottle indicated that the medium was saturated with lumazine. The lumazine-saturated medium was filter-sterilized, and 5, 2.5, and 1.25 mL were mixed with 0, 2.5, and 3.75 mL of previously filter sterilized medium, respectively, and injected into sealed, previously autoclaved Hungate tubes, to achieve 100, 50, and 25% lumazine-

saturated medium. Lumazine solubility could not be measured because it was difficult to establish the minimum saturating concentration.

Inoculation and incubation

Stock cultures were grown on MS medium (Boone et al., 1989) and 0.2 mL delivered into autoclaved Hungate tubes with MS medium. Tubes were then pressurized with O_2-free H_2 at 138 kPa. Tubes were then horizontally placed in a shaking incubator at 37 °C at 120 rpm.

Measurements and calculations

Methanogen growth can be followed by CH_4 production (Balch and Wolfe, 1976; Balch et al., 1979). At 4 and 6 d of incubation, tubes were analyzed for CH_4 production using a flame ionization-detection GC (Chong-Song et al., 2002). A 10% CH_4 gas mixture at 69 kPa was used as a standard. Micromoles of CH_4 produced per tube were calculated from its partial pressure and headspace volume.

Statistical analysis

The experimental model for each of the chemical additives was: CH_4 production = overall mean + species + concentration + day (4 or 6) + random effect of tube nested within species and concentration + species x concentration + species x day + concentration x day + species x concentration x day + residual. Concentration of BES, BPS, and lumazine (% of saturation) was log transformed (euler base). Type III mean squares for tube nested within species and concentration were used as the error term for

species and concentration effects and their interaction (Neter et al., 1996). Depending on the significance of triple and double interactions, linear and quadratic contrasts for concentration were evaluated separately for each species or each combination of species by day. If both linear and quadratic contrasts were significant ($P < 0.05$), a quadratic response was declared. Comparisons of interest between means of the same species and chemical concentration on different days were done by the Scheffe test (Neter et al., 1996). Due to heterogeneity of variances, the repeated/group statement of SAS was used to estimate separate variances per concentration for BES, per species for lumazine, and per combination of species and concentration for propynoic acid (SAS®, 1999).

Results and Discussion

BES

At 4 d, CH_4 production of *M. ruminantium* was decreased by BES at 10 μM and was minimal at 50 and 250 μM (quadratic contrast $P = 0.03$; Table 8-1). By day six, *M. ruminantium* had some recovery at 50 mM BES ($P < 0.01$; means, but not probability, are shown in Table 8-1). In contrast, *M. mazei* and *M. mobile* were minimally affected by BES at 10 or 50 μM (species x concentration; $P < 0.01$). *M. mobile* showed minimal CH_4 production with 250 μM BES (linear and quadratic contrast $P < 0.01$), while *M. mazei* was inhibited by 250 μM BES during the first 4 d, but showed a strong recovery by day six ($P < 0.01$; means, but not probability, are shown in Table 8-1). In agreement with the current results, methanogens resistant to BES have been found in sheep that were never fed the chemical (Ungerfeld, 1998).

Methanogens of the family Methanobacteriaceae, of which *M. ruminantium* is the main ruminal species (Sharp et al., 1998), were dominant in a protozoa-enriched fraction of ruminal fluid from a Holstein cow. On the contrary, the order Methanomicrobiales, of which *M. mobile* is the only ruminal species isolated, was negatively associated with the presence of protozoa (Sharp et al., 1998). *M. mobile* was also the dominant methanogen in sheep ruminal fluid in which few protozoa had ecto or endosymbiotic methanogens (Yanagita et al., 2000). Higher resistance to BES was sometimes found in defaunated compared to faunated ruminal fluid (Ungerfeld, 1998). This may be explained by a high proportion of *M. mobile* in total archaea in the absence of protozoa, as *M. mobile* had a greater resistance to BES compared to *M. ruminantium* in the present experiment.

Table 8-1. Effects of 2-bromoethanesulfonate (BES) on CH_4 production (μmoles) of three ruminal methanogens[1]

		BES concentration (μM)					
Species	Days	0	10	50	250	L[2]	Q[3]
Methanobrevibacter ruminantium	4	118	78	11	ND[4]	< 0.01	0.03
	6	111	96	31	ND	< 0.01	0.23
Methanosarcina mazei	4	135	131	131	13	< 0.01	< 0.01
	6	132	119	126	87	< 0.01	0.06
Methanomicrobium mobile	4	103	108	109	1.5	< 0.01	< 0.01
	6	101	107	106	5.7	< 0.01	< 0.01

[1]Species ($P < 0.01$); Concentration ($P < 0.01$); Days ($P < 0.01$); Tube (Species x Concentration; $P < 0.01$); Species by concentration ($P < 0.01$); Species by days ($P < 0.01$); Concentration by days ($P < 0.01$); Species by concentration by days ($P < 0.01$); SEM 0 μM = 0.32; SEM 10 μM = 1.24; SEM 50 μM = 1.74 μM; SEM 250 μM = 1.31; [2]Significance of linear contrast; [3]Significance of quadratic contrast; [4]ND = not detected.

BES is a structural analog of coenzyme M (CoM) and inhibits the reductive demethylation of methyl-S-CoM in the last step of methanogenesis (Müller et al., 1993). Resistance to BES in the non-ruminal *Methanococcus voltae* (Santoro and Konisky, 1987) and a non-ruminal species of *Methanosarcina* (Smith, 1983) was shown to be

based on an inability to transport BES into the cell. Inhibition of *M. ruminatium* (Balch and Wolfe, 1979a) and a non-ruminal *Methanosarcina* (Smith and Mah, 1981) by BES was lower when CoM concentration in the medium was elevated. Coenzyme M prevented the uptake of BES and protected *Methanococcus voltae* cells from BES inhibition (Santoro and Konisky, 1987). Methanogens that have the ability to synthesize CoM are less dependent on CoM taken from the medium. In a medium containing 5 μM CoM, *M. mobile* and *Methanobrevibacter smithii* (*M. ruminantium* PS) had 90% lower CoM uptake than *M. ruminantium* M1 (Balch and Wolfe, 1979b). Both *M. mobile* and *M. smithii* can synthesize CoM intracellularly (Balch and Wolfe, 1979b; Stewart et al., 1997). *M. ruminantium* M1, in contrast, requires CoM in the medium, and was shown in the current experiment to be highly sensitive to BES. *M. mazei*, which was more resistant to BES, has the ability to synthesize the CoM (Stewart et al., 1997). Methanogens that can synthesize CoM exhibit lower rates of transport of external CoM into the cell and are likely more resistant to BES. As the medium used in the present experiment contained around 1,200 μM CoM, the inhibition of *M. mobile* and *M. mazei* might have been greater with less or no added CoM.

M. ruminantium is the only ruminal methanogen isolated that requires CoM (Stewart et al., 1997). The implications would be that feeding BES could result in a selection of other methanogens that would occupy the empty niche left by *M. ruminantium*, causing BES-resistance of the mixed ruminal microbiota as observed in vivo (Van Nevel and Demeyer, 1996). The accumulation of H_2 observed when ruminal methanogenesis is inhibited (Van Nevel and Demeyer, 1996) could allow for faster growth rates of the non-inhibited, BES-resistant methanogens.

In the present experiment, *M. mazei* seemed to adapt to 250 μM BES between 4 and 6 d. In a previous report, BES at 150 μM inhibited methanol utilization in sheep ruminal fluid 1 h after its addition, indicating that the chemical was toxic for *Methanosarcina* at that concentration (Pol and Demeyer, 1988); however, it was not reported whether the inhibition remained over a longer period. There are, nevertheless, previous reports on spontaneous acquisition of resistance to BES in *Methanosarcina* spp. (Smith and Mah, 1981). The non-ruminal methanogen *Methanosarcina* strain 227 was inhibited by 24 μM BES, but spontaneously resumed growth after 6 d. It was shown that the acquisition of resistance was based on heritable changes and was not due to biological inactivation of BES, or inactivation through reaction with medium components. Interestingly, BES resistance was retained even after 30 transfers in the absence of BES (Smith and Mah, 1981). In the present experiment, *M. ruminantium* also showed some adaptation between 4 and 6 d.

It is apparent , therefore, that BES adaptation of mixed ruminal microbiota (Van Nevel and Demeyer, 1996) is based on at least two mechanisms: selection of species of methanogens with greater resistance to BES, and selection of BES-resistant strains or mutants within species. It is also possible that BES degradation by the mixed ruminal microbiota occurs. Reduction of BES sulfonic moiety to sulfide, presumably by sulfate-reducing bacteria, has been reported for the microbial community of a river sediment (Ye et al., 1999).

BPS

BPS did not affect CH_4 production in any of the species studied ($P = 0.17$; Table 8-2). Previously, methanogenesis of a mixed ruminal culture was shown to be unaffected by BPS (Ungerfeld et al., 2002). BPS, however, has been found to be a very potent inhibitor of methyl-CoM reductase in a pure enzyme-substrate system (Ellerman et al., 1989). The opposite results observed with the purified enzyme-substrate system and with pure methanogen and mixed cell cultures, suggest that BPS is not transported into cells. BPS has a closer structure to methyl-CoM than BES (Ellerman et al., 1989). Coenzyme M and BES have been shown to share a common transport system (Smith, 1983; Santoro and Konisky, 1987). Coenzyme M transport system may not be suitable for transporting methyl-CoM or BPS into the cell.

Table 8-2. Effects of 3-bromopropanesulfonate (BPS) on CH_4 production (μmoles) of three ruminal methanogens[1]

		BPS concentration (μM)			
Species	Days	0	10	50	250
Methanobrevibacter ruminantium	4	118	132	127	133
	6	111	123	121	124
Methanosarcina mazei	4	135	132	128	134
	6	132	130	125	132
Methanomicrobium mobile	4	103	108	110	109
	6	101	106	107	105

[1]Species ($P < 0.01$); Concentration ($P = 0.17$); Days ($P < 0.01$);
Tube (Species x Concentration; $P < 0.01$); Species x Concentration ($P = 0.19$);
Species x Days ($P < 0.01$); Days x Concentration ($P = 0.69$);
Species x Concentration x Days ($P = 0.41$); SEM = 1.19.

Lumazine

For the three species studied, CH_4 production in a medium that was 50 or 100% saturated with lumazine was minimal ($P < 0.01$; Table 8-3). Inhibition was less at 25% lumazine saturation. *M. ruminantium* increase in CH_4 production at 25% lumazine saturation by d 6 was non-significant ($P = 0.91$; means, but not probability, are shown in Table 8-3).

Table 8-3. Effects of lumazine on CH_4 production (μmoles) of three ruminal methanogens[1]

		Concentration (% saturation)					
Species	Days	0	25	50	100	L[2]	Q[3]
Methanobrevibacter ruminantium	4	118	42	4.6	0.8	< 0.01	< 0.01
	6	111	84	4.6	0.7	< 0.01	0.23
Methanosarcina mazei	4	135	24	7.8	0.6	< 0.01	< 0.01
	6	132	33	7.6	0.4	< 0.01	< 0.01
Methanomicrobium mobile	4	103	27	2.4	ND[4]	< 0.01	< 0.01
	6	101	26	2.3	ND	< 0.01	< 0.01

[1]Species ($P < 0.01$); Concentration ($P < 0.01$); Days ($P = 0.14$);
Tube (Species x Concentration; $P = 0.01$); Species x Concentration ($P < 0.01$);
Species x Days ($P = 0.07$); Days x Concentration ($P < 0.01$);
Species x Days x Concentration ($P < 0.01$); SEM *M. ruminantium* = 2.89;
SEM *M. mazei* = 0.60; SEM *M. mobile* = 0.08; [2]Significance of linear contrast;
[3]Significance of quadratic contrast; [4]ND = not detected.

Lumazine concentration in the 50 and 100% lumazine-saturated media was lower than 0.12 and 0.24 mM, respectively. However, the inhibition achieved was much greater than what has been observed in mixed cultures with 1.2 mM lumazine (Chapters 4 and 6). It is possible that microbial species present in mixed cultures took up lumazine and prevented it from inhibiting methanogens. Adsorption of lumazine to solid particles could perhaps prevent its uptake by methanogens. Also, solid particles harbor a dense adhered microbial population (Craig et al., 1987; Olubobokun and Craig, 1990), that may accelerate lumazine catabolism, if it occurred.

Propynoic acid

Propynoic acid at 2 mM was strongly inhibitory for *M. ruminantium* and *M. mobile*, and less inhibitory for *M. mazei* ($P < 0.01$; Table 8-4). At 4 mM, propynoic acid caused further inhibition of *M. ruminantium*, but had minimal further effects on *M. mazei* or *M. mobile* ($P < 0.01$). At both concentrations, inhibition of CH_4 production of *M. ruminantium* and *M. mobile* was greater than previously observed with ruminal mixed cultures (Chapter 6). As *M. ruminantium* and *M. mobile* are generally the dominant ruminal methanogens (Lin et al., 1997; Sharp et al., 1998), it would be expected that propynoic acid inhibition of methanogenesis in mixed ruminal cultures would follow the pattern observed for these two species in pure culture. As little propynoic acid disappeared (Chapters 3 and 6), it is unlikely that the lower inhibition of methanogenesis observed with mixed cultures was due to propynoic acid metabolism by other ruminal microorganisms. It is also improbable that the lower inhibition of methanogenesis observed in mixed cultures was due to propynoic acid adsorption to solid particles, as propynoic acid is highly hydrophilic. *M. mazei* was more resistant to propynoic acid than the two other species examined. Other species not studied herein, like *Methanosarcina barkeri*, could be more resistant to propynoic acid and perhaps explain the lower inhibition of CH_4 production observed with mixed cultures. Importantly, no adaptation to propynoic acid by any of the three species occured.

Table 8-4. Effects of propynoic acid on CH_4 production (μmoles) of three ruminal methanogens[1]

		Concentration (mM)				
Species	Days	0	2	4	L[2]	Q[3]
Methanobrevibacter ruminantium	4	118	11	3.9	< 0.01	< 0.01
	6	111	12	4.2		
Methanosarcina mazei	4	135	35	33	< 0.01	< 0.01
	6	132	33	31		
Methanomicrobium mobile	4	103	12	8.6	< 0.01	< 0.01
	6	101	11	8.5		

[1]Species ($P < 0.01$); Concentration ($P < 0.01$); Days ($P = 0.17$); Tube (Species x Concentration; $P = 0.31$); Species x Concentration ($P < 0.01$); Species x Days ($P = 0.93$); Concentration x Days ($P = 0.53$); Species x Concentration x Days ($P = 0.49$); SEM *M. ruminantium* 0 mM = 0.43; SEM *M. ruminantium* 2 mM = 0.34; SEM *M. ruminantium* 4 mM = 0.55; SEM *M. mazei* 0 mM = 0.77; SEM *M. mazei* 2 mM = 0.86; SEM *M. mazei* 4 mM = 0.65; SEM *M. mobile* 0 mM = 2.15; SEM *M. mobile* 2 mM = 0.050; SEM *M. mobile* 4 mM = 0.32 [2]Significance of linear contrast; [3]Significance of quadratic contrast.

Previous experiments with mixed ruminal cultures had found an increase in H_2 accumulation when methanogenesis was inhibited by propynoic acid (Chapters 3 and 6). This suggested that CH_4 production was not being inhibited through competition for electrons to reduce propynoic acid triple bond, as was originally hypothesized. The present results support that conclusion, as CH_4 production was inhibited at H_2 partial pressures 500 to 5,000 greater than those found in the rumen (Hungate, 1967; Kohn and Boston, 2000). Furthermore, the complete reduction of propynoic acid triple bond at 4 mM concentration would have demanded 4×10^{-5} moles of H_2, equivalent to approximately 3.5% of the H_2 originally present in the headspace. Thus, even if propynoic acid's triple bond had been completely reduced, methanogenesis would have still been amply thermodynamically favorable. The inhibition exhorted by propynoic acid, must be, therefore, non-competitive.

Ethyl 2-butynoate

Methane production of *M. ruminantium* was almost completely inhibited at both concentrations of ethyl 2-butynoate. *M. mazei* was resistant to this chemical at the concentrations studied. *M. mobile* was not affected by ethyl 2-butynoate at 4 mM, but almost completely inhibited at 8 mM (*M. mobile* quadratic contrast for days 4 and 6 $P <$ 0.01; Table 8-5).

Table 8-5. Effects of ethyl 2-butynoate on CH_4 production (μmoles) of three ruminal methanogens[1]

		Concentration (mM)				
Species	Days	0	4	8	L[2]	Q[3]
Methanobrevibacter ruminantium	4	118	0.4	ND[4]	< 0.01	< 0.01
	6	111	0.7	0.2	< 0.01	< 0.01
Methanosarcina mazei	4	135	129	134	0.78	0.14
	6	132	127	130	0.53	0.27
Methanomicrobium mobile	4	103	104	0.2	< 0.01	< 0.01
	6	101	101	1.2	< 0.01	< 0.01

[1]Species ($P < 0.01$); Concentration ($P < 0.01$); Days ($P < 0.01$); Species x Concentration ($P < 0.01$); Tube (Species x Concentration; $P < 0.01$); Species x Days ($P = 0.05$); Concentration x Days ($P < 0.01$); Species x Concentration x Days ($P < 0.01$); SEM = 1.02; [2]Significance of linear contrast; [3]Significance of quadratic contrast; [4]ND = not detected.

Cell envelope differences could be related to the differences observed in toxicity to ethyl 2-butynoate. The presence of an S-layer in *M. mazei* and *M. mobile*, which is absent in *Methanobrevibacter* spp. (Sprott and Beveridge, 1993), may confer the former some resistance to ethyl 2-butynoate. The methanochondroitin layer of *M. mazei* (Sprott and Beveridge, 1993) might confer this organism some additional protection to resist a higher concentration of 8 mM ethyl 2-butynoate. Also, *Methanosarcina* aggregates in large clumps (Pol and Demeyer, 1988; Moss, 1993) that may restrain the access of ethyl 2-butynoate to cells. Microscopic observations, however, did not find *Methanosarcina* clump formation in the present experiment (not shown).

Resistance of *M. mazei* to ethyl 2-butynoate can be a problem for its practical use in vivo. If *M. mazei* or other *Methanosarcina* are completely resistant to this chemical, its long term use may preferentially select for them, and the inhibition of CH_4 production dissipate as the proportions of methanogens change.

The same rationale for the nature of inhibition of CH_4 formation with propynoic acid applies to ethyl 2-butynoate. At 8 mM initial concentration, the complete reduction of ethyl 2-butynoate triple bond would have implied the utilization of approximately 7% of H_2 initially present in the headspace. Even if ethyl 2-butynoate triple bond had been completely reduced, methanogenesis would still be largely thermodynamically favorable. Other hypothetical reductions that this molecule could undergo (e.g., keto group in the ester bond) would have little quantitative significance as a H sink in this experiment. One must conclude that, as with propynoic acid, the inhibition exhorted by ethyl 2-butynoate is non-competitive. Triesters such as trilinolein or trilinolenin at 10 mM have been shown to be non-toxic to *Methanobrevibacter* (*Methanobacterium*) *ruminantium* (Prins et al., 1972). Perhaps the toxicity of ethyl 2-butynoate to methanogens involves its a smaller molecule size compared to those triglycerides, and/or to the presence of the triple bond.

Conclusions

There were important differences among the three ruminal methanogens examined with regard to their sensitivity to some of the compounds investigated. *M. ruminantium* was the most sensitive species to BES, propynoic acid, and ethyl 2-butynoate, *M. mazei* was the least sensitive species to those three chemicals, and *M.*

mobile was intermediate. Conversely, *M. ruminantium* was the least sensitive species to lumazine. Differences among species in resistance to chemicals can have important practical implications as they may result in the selection of the more resistant species. Adaptation of the ruminal microbiota to BES was found after 3 d of feeding this chemical to sheep (Van Nevel and Demeyer, 1996). This was likely related to differences among methanogens regarding their sensitivity to the inhibitor. There is a need to monitor long term changes in the populations of different methanogens when inhibitors of methanogenesis are administrated. 16S rRNA probes could be useful for this purpose (Lin et al., 1997; Sharp et al., 1998), especially to study unculturable organisms (Yanagita et al., 2000; Tajima et al., 2001).

There seemed to be adaptation by the pure cultures to some of the chemicals. *M. mazei* seemed to adapt to 250 mM BES after 6 d. *M. ruminantium* showed some recovery at 50 mM BES between 4 and 6 d. Mechanisms of action of the inhibitors, and of subsequent adaptation of individual species of methanogens, need to be studied before in vivo strategies to inhibit ruminal methanogenesis can be developed.

Perhaps future in vivo strategies could be based on combinations or rotations of different inhibitors of methanogenesis. Possibly, lumazine or propynoic acid could be used to specifically target *Methanosarcina* and combined with other inhibitors to which *Methanosarcina* is more resistant.

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CONCLUSIONS

None of the compounds or combinations of compounds studied substantially decreased CH_4 production without having some adverse effects on fermentation. However, those effects were milder at low concentrations of the inhibitors, and there were some unexpected beneficial effects such as decreased proteolysis and increased microbial N production.

It was hypothesized that the inhibition of pyruvate oxidative decarboxylation would result in less CO_2 and H_2 available for methanogenesis (Chapter 2). Inhibition of pyruvate oxidoreductases was attempted directly and through the inhibition of thiamine utilization, a cofactor of this reaction. Inhibition achieved in CH_4 production was small or non-existent. If further work in this line is to be considered, basic research on thiamine uptake and metabolism in ruminal microorganisms and the enzymology of their pyruvate oxidoreductases would be needed.

Alternative electron sinks to methanogenesis were examined (Chapter 3). Four compounds, oxaloacetate, acetoacetate, ß-hydroxybutyrate, and crotonate, are naturally occuring intermediates in ruminal fermentation. Another four compounds, propynoic acid, 3-butenoic acid, 2-butynoic acid, and ethyl 2-butynoate, are unsaturated analogs of VFA or other compounds. Propynoic acid and ethyl 2-butynoate were strong inhibitors of methanogenesis. However, fermentation also was reduced and fermentation products without a nutritional value accumulated. It was apparent that methanogenesis was inhibited directly, rather than through competition for reducing equivalents. ß-

Hydroxybutyrate, crotonate, and 3-butenoic acid, in contrast, had minimal effects on CH_4 production, but seemed to stimulate fermentation.

Three novel compounds with unrelated mechanisms of inhibition of CH_4 formation were studied in Chapter 4. The archaeal DNA-polymerase inhibitor aphidicolin did not affect CH_4 production. A pterin, lumazine, decreased CH_4 formation by about 50%, but also decreased apparent OM fermentation from 43.5 to 36.1%. Surprisingly, H_2 accumulation was not observed and acetate molar percentage increased. Lumazine inhibited the reduction of methyl-CoM to CH_4 in the last step of methanogenesis (Nagar-Anthal and Nagle, 1997). The methyl-CoM analog 2-bromopropanesulfonate (BPS) did not affect CH_4 formation.

The effects of a novel hexadecatrienoic acid extracted from a marine algae and olive oil on CH_4 production and fermentation were investigated in Chapter 5. The hexadecatrienoic acid decreased CH_4 production by 97%, but decreased apparent OM fermentation and increased H_2 accumulation. Olive oil did not affect CH_4 production or fermentation, but increased propionate and butyrate production, and tended to decrease acetate production. Perhaps olive oil could be used to increase dietary energy and glucose supply without negatively affecting fermentation.

Some of the compounds studied in Chapters 2 through 5 decreased CH_4 production considerably, but had other undesirable effects. Previous reports have shown that other inhibitors of CH_4 production result in an increased accumulation of nutritionally non-usable products like H_2, formate, and ethanol and decrease fermentation (Van Nevel and Demeyer, 1996). These problems are a general consequence of an inefficient relocation of the electrons spared from CH_4 formation (Van Nevel and

Demeyer, 1996). The organic acids that seemed to stimulate fermentation in Chapter 3 could have acted as H sinks. Consequently, it was hypothesized that they could relieve the negative effects on fermentation of the inhibitors of CH_4 formation. Three inhibitors of CH_4 formation, lumazine, propynoic acid, and ethyl 2-butynoate, and two organic acids that stimulated fermentation, crotonic acid, and 3-butenoic acid, were selected for further investigation. The effects on fermentation of combinations between lumazine, propynoic acid or ethyl 2-butynoate, and crotonic acid or 3-butenoic acid were examined in Chapter 6. The effects of those combinations on OM, N, and NDF degradation and microbial OM and N production were examined in Chapter 7.

Resistance of individual species of methanogens to chemical inhibitors could lead to adaptation of the ruminal microbiota, and difficulties to maintain long term inhibition of CH_4 production (Ungerfeld, 1998). The effects of lumazine, propynoic acid, ethyl 2-butynoate, 2-bromoethanesulfonate (BES) and BPS, on pure cultures of three ruminal methanogens were examined in Chapter 8.

Lumazine

Inhibition of CH_4 formation obtained with lumazine has been inconsistent, ranging from 9 (Chapter 6) to 50% (Chapter 4). Ruminal fluid donors and substrate used were similar in the experiments reported. However, the numbers of different species of methanogens could have changed throughout time as experiments in Chapters 4 and 6 were more than one year apart. Previous observations with sheep suggested that the numbers of methanogens resistant and sensitive to BES changed throughout time in animals whose diet and management remained constant (Ungerfeld, 1998). Differences

in the proportions of methanogens in ruminal fluid may be responsible for the variable results, as resistance to lumazine (measure through CH_4 production by monocultures) was shown to vary among species. *Methanobrevibacter ruminantium* was shown to be more resistant to lumazine than *Methanosarcina mazei* or *Methanomicrobium mobile* (Chapter 8).

Other effects of lumazine as an inhibitor of ruminal CH_4 formation have been peculiar. No increase in unusual reduced end products of fermentation (H_2, formate, or ethanol) were observed, which indicates that metabolic H was efficiently relocated into other pathways. This cannot be totally explained by the relatively mild inhibition of CH_4 formation observed with lumazine. A 50% decrease in CH_4 formation caused by lumazine (Chapter 4) was not accompanied by any increase in H_2, formate or ethanol accumulation, while similar degrees of inhibition caused by intermediate concentrations of propynoic acid or ethyl 2-butynoate resulted in increases in these products (Chapters 3 and 6). Although apparent OM fermentation (estimated through a mass balance) decreased with lumazine addition (Chapter 4), it was then shown that true OM degradation was unaffected (Chapter 7). A decrease in OM apparent fermentation without changes in true degradation suggests a shift of OM sinks from fermentation products to microbial biomass; however, the proportion of degraded OM incorporated into microbial biomass was largely unaffected by lumazine (Chapter 7). Like the accumulation of unusual reduced end products of fermentation such as H_2, formate and ethanol, a decrease in fiber degradation is another consequence of changes in electron disposal and cofactor re-oxidation when methanogenesis is inhibited (Van Nevel and Demeyer, 1996). The lack of decrease of truly degraded OM (Chapter 7) again suggests

that the inhibition of CH_4 formation by lumazine somehow did not create an electron disposal problem.

It is also of interest that lumazine was a much stronger inhibitor of CH_4 formation in pure cultures of methanogens (Chapter 8) than in mixed ruminal cultures (Chapters 4 and 6). It seems likely that in mixed ruminal cultures lumazine was taken up and metabolized. Decreases in N degradation and large changes in isobutyrate production suggest that lumazine also affected non-methanogens. This is contrary to a previous report, where there were no effects of lumazine on growth of non-ruminal bacteria (Nagar-Anthal et al., 1996). The effects of lumazine would need to be evaluated in pure cultures of ruminal non-methanogens, and changes in the numbers of different microbial groups in mixed ruminal cultures would need to be assessed.

It could be worthwhile to do further research on the effects of lumazine on ruminal fermentation and animal production. From the applied nutrition point of view, lumazine could cause some decrease in CH_4 production in the rumen, and thus increase the retention of fermented energy into usable products. It also may decrease ruminal proteolysis and NH_4^+ concentration and increase microbial N production (Chapter 7). Lumazine may have potential to increase the retention of dietary energy and N into usable products. This may have beneficial environmental consequences, such as less CH_4 and N released into the environment. The toxicological properties of lumazine have not been investigated (MSDS, 2000), so its toxicity to ruminants and the environment would need to be studied.

It is of great interest to understand why lumazine does not cause the problems in the relocation of electrons that have been observed with other inhibitors of ruminal

methanogenesis. Lumazine increased (Chapter 4 and 6) or did not affect (Chapter 6) the acetate to propionate ratio, which is contrary to what has been observed with other inhibitors of ruminal methanogenesis (Nagaraja et al., 1997). Stimulation of reductive acetogenesis by lumazine could perhaps explain the shift towards acetate, the lack of increase in H_2 accumulation, and the decrease in CO_2 release sometimes observed (Ungerfeld et al., 2002). It would be of interest to study the effects of lumazine on ruminal acetogens.

A lumazine derivative, 6, 7-dimethyl-8-ribityllumazine, is a precursor of riboflavin (Weimar and Neims, 1975). Riboflavin is in turn a precursor of the electron acceptor FAD (Voet and Voet, 1995). The inhibition of CH_4 formation with its consequential disruption of the interspecies H transfer might elevate the requirements of the mixed ruminal microbiota for riboflavin, and perhaps also nicotinamide, an NAD precursor. This would be another potential area for further research.

Propynoic acid

Propynoic acid was a strong inhibitor of ruminal methanogenesis in vitro. Contrary to what was hypothesized originally, propynoic acid did not decrease CH_4 formation by capturing reducing equivalents, but inhibited methanogens directly (Chapter 8). Propynoic acid had undesirable effects on degradation and increased the accumulation of nutritionally non-usable fermentation products. Accumulation of these products was not relieved by crotonic acid or 3-butenoic acid. 3-Butenoic acid could mitigate the negative effects of propynoic acid on OM degradation (Chapters 6 and 7).

Since propynoic acid is moderately toxic to rodents and is not readily metabolized, its practical utilization in ruminant diets is difficult. However, propynoic acid caused substantial increases in microbial OM and N production, due to considerable improvements in microbial synthetic efficiency (Chapter 7). It is of great interest to understand how propynoic acid addition increased the efficiencies of microbial OM and N synthesis. As little additive disappeared, these improvements were not caused by the utilization of the additive as a C or energy source. If propynoic acid was deleterious to protozoa, the improvement in microbial synthetic efficiency could be a consequence of less intrarruminal N recycling due to less protozoal predation on bacteria (Williams and Coleman, 1997). The decrease in VFA and NH_4^+ concentrations caused by propynoic acid would be consistent with an inhibition of protozoa (Williams and Coleman, 1997). However, lower VFA concentrations also could be a consequence of the lower OM degradation, and lower NH_4^+ concentrations could be a consequence of the higher production of microbial N observed with propynoic acid. Also, propynoic acid at 2 mM greatly improved microbial efficiencies of OM and N synthesis, but was not particularly inhibitory of butyrate production (Chapter 6). As protozoa are strong butyrate producers (Huhtanen, 1992; Jaakkola and Huhtanen, 1993), this may suggest that protozoa were unaffected by propynoic acid. Protozoal numbers should be monitored to assess the effects of propynoic acid.

Alternatively, propynoic acid may have inhibited deamination (Chapter 6). An increase in the supply of preformed amino acids can improve microbial efficiency (Leng and Nolan, 1984). Nevertheless, beneficial effects of amino acids occur with rapidly degradable energy sources (Wallace et al., 1997), and there might be no benefit with

slowly degradable substrates as used in this study. Regardless, if protozoal activity or lower deamination are not involved in the increased microbial synthetic efficiencies, it would be very important to understand how propynoic acid caused these improvements, and how this could be applied to manipulate ruminal fermentation.

Ethyl 2-butynoate

Similarly to propynoic acid, ethyl 2-butynoate was a strong inhibitor of ruminal methanogenesis in vitro. It did not decrease CH_4 formation by withdrawing reducing equivalents, but inhibited methanogens directly (Chapter 8). Ethyl 2-butynoate caused the accumulation of nutritionally non-usable fermentation products, which was not relieved by crotonic acid or 3-butenoic acid. However, ethyl 2-butynoate at an initial concentration of 4 mM did not decrease OM and NDF degradation. Furthermore, it strongly increased microbial OM and N production through increased efficiencies of synthesis. However, when the additive's disappearance was considered, synthetic efficiencies were unchanged.

The inclusion of ethyl 2-butynoate in ruminant diets to provide average ruminal concentrations of approximately 4 mM (or slightly lower, as average concentrations in vitro were lower than initial due to disappearance) could have an applied nutrition interest. In vitro degradation of OM was not affected by 4 mM initial concentration of ethyl 2-butynoate. Furthermore, in vivo small decreases in ruminal degradation could be compensated post ruminally. Ethyl 2-butynoate at 4 mM could decrease CH_4 production by about 50%, decrease ruminal proteolysis and increase microbial N flow. This could result in improvements in N retention and reduce N voided to the environment in feces

and urine. Ethyl 2-butynoate toxicity has not been investigated (GFS Chemicals, 1998), but it is advantageous that it completely disappeared by 24 h of incubation. This would decrease risks for the animal or the environment. The greatest problem to resolve, however, would be to relieve the accumulation of H_2, formate, and ethanol. Effectiveness of the relocation of electrons spared from methanogenesis into nutritionally useful electron sinks needs to be improved, either by using new electron acceptors, or by developing new strategies to better utilize the electron acceptors already studied.

The negative effects of H_2 accumulation on fermentation could be milder in vivo, if the animal could release the increased gas pressure more frequently. Hydrogen released to the atmosphere could perhaps escape the earth without causing the deleterious effects that CH_4 does.

Resistance of *M. mazei* to 8 mM ethyl 2-butynoate and of *M. mobile* to 4 mM (Chapter 8) can be a problem for their potential use in vivo. If some methanogens are resistant to this chemical, it is conceivable that long term use of ethyl 2-butynoate could select the resistant organisms and lead to adaptation of the microbial community to the chemical, as it has been found with BES (Van Nevel and Demeyer, 1996). Ethyl 2-butynoate could be combined with lumazine, which exhorts a better control on *M. mazei* and *M. mobile*.

Toxicity of ethyl 2-butynoate to ruminants and the environment, and potential problems with its practical application also would need to be addressed.

Crotonic acid, 3-butenoic acid, and other electron acceptors

The inhibition of CH_4 formation disrupts the interspecies H transfer and causes the formation of end products without a nutritional value. It was hypothesized that the addition of an external electron acceptor could solve this problem. However, both crotonic acid and 3-butenoic acid were largely ineffective in decreasing the accumulation of H_2, formate, or ethanol (Chapter 6).

It was found that crotonic acid and 3-butenoic acid stimulated the substrate apparent OM fermentation, as estimated through a mass balance (Chapter 3). Thus, it was hypothesized that these organic acids could relieve the constraints on fermentation caused by the inhibitors of CH_4 formation. Crotonic acid was ineffective in relieving the decreased OM and NDF degradation caused by propynoic acid or ethyl 2-butynoate (Chapter 7). 3-Butenoic acid relieved some of the negative effects on OM and NDF degradation caused by propynoic acid. Overall, the effects of crotonic acid and 3-butenoic acid on true OM degradation were much smaller than initially observed on apparent OM fermentation in Chapter 3. The combination of crotonic acid with lumazine, and 3-butenoic acid with propynoic acid at 2 mM, decreased the proportion of disappeared OM incorporated into microbial biomass. As true OM degradation was unaffected, apparent OM degradation would increase. Although this mechanism can explain why the increases in OM fermentation were not repeated for these two combinations of inhibitors and organic acids, it does not apply to other combinations or to crotonic acid and 3-butenoic acid overall. In addition, increased substrate apparent OM fermentation was not consistently observed in Chapter 6 (data not shown): crotonic acid increased ($P < 0.01$) the substrate apparent OM fermentability when combined with

lumazine, but not with propynoic acid or ethyl 2-butynoate. 3-Butenoic acid increased (P = 0.04) the substrate apparent OM fermentability only when combined with ethyl 2-butynoate.

It was a constant pattern throughout this work that externally added fermentation intermediates were not completely converted to the fermentation pathways end products. Most of added oxaloacetate did not seem to be converted to propionate, but to acetate. Important amounts of the butyrate enhancers acetoacetate, ß-hydroxybutyrate, and crotonate also seemed to be converted to acetate (Chapter 3). Similar results have been reported before for other organic acids (López et al., 1999). These unexpected results can be understood on thermodynamic grounds. Fermentation intermediates are normally present at very low concentrations. Yet, their conversion into the next compound in the pathway is still thermodynamically favorable, because the ΔG_0 of the reaction and the concentration of the next intermediate in the pathway are sufficiently low. Reverse reactions are thermodynamically unfeasible under normal conditions. However, artificially elevated concentrations of a fermentation intermediate normally present at very low concentrations could reverse the direction of the reaction.

One approach to resolve this problem would be to add the compounds continuously, so that their concentration at any time would be only slightly elevated. Thus, it may be more appropriate to do research on alternative electron acceptors in continuous culture than in batch culture. Experiments done in the chemostat would more closely reproduce events in the rumen when animals consume several meals per day.

However, the thermodynamic threshold for the reverse reaction of a pathway may still be too low in terms of the added compound concentration. A second approach to

address this problem would be to combine intermediates of different pathways to achieve the same molarity as with a single compound. This would allow lower concentrations of each externally added compound. While keeping reverse reactions thermodynamically unfavorable, it would still provide potential electron acceptors. Electron acceptors that are not natural fermentation intermediates could also be added if conversion into other compounds of the pathway does not occur. For example, if no isomerization occurs, a 50:50 molar ratio solution of 3-butenoic acid and crotonic acid would in theory be more efficiently converted to butyrate than each compound added separately at a similar molarity. Experiments with tracers would be needed to study how the concentration of externally added fermentation intermediates affects the proportion that is converted to the pathway end product.

Reductive acetogenesis could be an interesting alternative to rechannel electrons into acetate formation, because it does not involve the addition of a metabolite at high concentrations. Under normal conditions, H_2 pressure in the rumen is too low for reductive acetogenesis to compete with methanogenesis (Kohn and Boston, 2000). However, with the increase in H_2 partial pressure caused by the inhibition of CH_4 production, reductive acetogenesis could become thermodynamically favorable, and H_2 used to form acetate (Nollet et al., 1997).

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APPENDICES

Table A-1 Unprocessed data for Chapter 2, Experiment 1: optical density

Thiamine (mM)	Amprolium (mM)	Intracelllular inhibitor	OD[1] 0 h	OD 6 h	OD 12 h	OD 18 h	OD 24 h
10	10	nothing	0.247	0.462	0.388	0.337	0.34
10	10	nothing	0.255	0.424	0.378	0.345	0.337
10	10	nothing	0.257	0.388	0.331	0.308	0.306
10	10	nothing	0.242	0.408	0.38	0.327	0.332
10	10	nothing	0.245	0.465	0.388	0.354	0.366
10	10	nothing	0.258	0.461	0.327	0.312	0.33
10	0	nothing	0.268	0.331	0.276	0.275	0.323
10	0	nothing	0.27	0.387	0.283	0.258	0.278
10	0	nothing	0.47	0.398	0.375	0.388	0.363
10	0	nothing	0.276	0.38	0.306	0.287	0.3
10	0	nothing	0.25	0.378	0.284	0.278	0.306
10	0	nothing	0.255	0.423	0.375	0.367	0.373
0	10	nothing	0.282	0.427	0.377	0.293	0.302
0	10	nothing	0.277	0.479	0.379	0.306	0.3
0	10	nothing	0.32	0.435	0.331	0.306	0.311
0	10	nothing	0.299	0.39	0.343	0.299	0.312
0	10	nothing	0.276	0.405	0.321	0.287	0.299
0	10	nothing	0.284	0.468	0.338	0.304	0.31
0	0	nothing	0.427	0.337	0.281	0.381	0.463
0	0	nothing	0.261	0.299	0.269	0.28	0.283
0	0	nothing	0.252	0.374	0.278	0.267	0.29
0	0	nothing	0.428	0.378	0.367	0.347	0.344
0	0	nothing	0.248	0.345	0.32	0.317	0.312
0	0	nothing	0.273	0.43	0.379	0.389	0.341

[1]OD = optical density.

Table A-1 (cont'd)

Thiamine (mM)	Amprolium (mM)	Intracelllular inhibitor	OD[1] 0 h	OD 6 h	OD 12 h	OD 18 h	OD 24 h
10	10	Adenine	0.349	0.308	0.28	0.263	0.25
10	10	adenine	0.323	0.291	0.289	0.259	0.247
10	10	adenine	0.246	0.338	0.342	0.254	0.249
10	10	adenine	0.257	0.346	0.323	0.264	0.257
10	10	adenine	0.23	0.311	0.307	0.255	0.248
10	10	adenine	0.242	0.319	0.286	0.239	0.233
10	0	adenine	0.252	0.316	0.273	0.234	0.225
10	0	adenine	0.236	0.421	0.266	0.212	0.207
10	0	adenine	0.24	0.357	0.263	0.228	0.234
10	0	adenine	0.257	0.308	0.272	0.235	0.222
10	0	adenine	0.242	0.346	0.285	0.261	0.232
10	0	adenine	0.263	0.368	0.278	0.229	0.244
0	10	adenine	0.263	0.374	0.338	0.257	0.248
0	10	adenine	0.286	0.362	0.321	0.286	0.267
0	10	adenine	0.607	0.434	0.4	0.353	0.373
0	10	adenine	0.272	0.353	0.316	0.258	0.244
0	10	adenine	0.268	0.395	0.308	0.267	0.235
0	10	adenine	0.32	0.377	0.328	0.28	0.261
0	0	adenine	0.275	0.41	0.281	0.229	0.218
0	0	adenine	0.271	0.385	0.298	0.256	0.228
0	0	adenine	0.271	0.446	0.34	0.314	0.261
0	0	adenine	0.276	0.361	0.287	0.248	0.232
0	0	adenine	0.268	0.392	0.319	0.242	0.236
0	0	adenine	0.263	0.401	0.323	0.28	0.267
10	10	adenosine	0.258	0.413	0.527	0.848	0.648
10	10	adenosine	0.247	0.374	0.52	0.858	0.643
10	10	adenosine	0.267	0.405	0.521	0.83	0.638
10	10	adenosine	0.258	0.496	0.582	0.884	0.718

[1]OD = optical density.

Table A-1 (cont'd)

Thiamine (mM)	Amprolium (mM)	Intracelllular inhibitor	OD[1] 0 h	OD 6 h	OD 12 h	OD 18 h	OD 24 h
10	10	adenosine	0.262	0.439	0.537	0.948	0.704
10	10	adenosine	0.258	0.451	0.552	0.84	0.708
10	0	adenosine	0.251	0.443	0.51	0.868	0.708
10	0	adenosine	0.255	0.429	0.54	0.858	0.666
10	0	adenosine	0.247	0.486	0.553	0.812	0.724
10	0	adenosine	0.245	0.394	0.499	0.86	0.704
10	0	adenosine	0.244	0.345	0.5	0.84	0.68
10	0	adenosine	0.255	0.508	0.555	0.876	0.764
0	10	adenosine	0.274	0.52	0.672	0.88	0.687
0	10	adenosine	0.27	0.457	0.568	0.88	0.653
0	10	adenosine	0.268	0.477	0.561	0.903	0.679
0	10	adenosine	0.279	0.42	0.554	0.902	0.704
0	10	adenosine	0.275	0.462	0.603	0.912	0.724
0	10	adenosine	0.274	0.487	0.567	0.918	0.756
0	0	adenosine	0.279	0.433	0.554	0.83	0.642
0	0	adenosine	0.319	0.39	0.491	0.958	0.782
0	0	adenosine	0.273	0.432	0.546	0.867	0.68
0	0	adenosine	0.273	0.453	0.559	0.908	0.75
0	0	adenosine	0.281	0.496	0.622	0.91	0.808
0	0	adenosine	0.266	0.438	0.631	0.904	0.714

[1]OD = optical density.

Table A-2 Unprocessed data for Chapter 2, Experiment 1: gases, fermentation, and ammonia

Thiamine (mM)	Amprolium (mM)	Intracelllular inhibitor	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM, µmoles/mg	NH_4^+, ppm
10	10	nothing	3.47	1645	7.1	0.21	171
10	10	nothing	6.77	284	8.7	0.34	175
10	10	nothing	4.38	211	7.2	0.27	166
10	10	nothing	4.19	289	7.3	0.25	162
10	10	nothing	5.14	235	8.5	0.26	152
10	10	nothing	6.71	241	9.4	0.31	165
10	0	nothing	7.09	242	10.2	0.39	169
10	0	nothing	7.35	267	9.6	0.43	174
10	0	nothing	16.37	1741	14.5	0.64	232
10	0	nothing	9.52	255	9.8	0.55	203
10	0	nothing	9.58	276	9.6	0.56	180
10	0	nothing	11.54	259	9.4	0.69	177
0	10	nothing	12.45	287	9.2	0.78	190
0	10	nothing	6.67	246	9.8	0.39	194
0	10	nothing	7.65	249	10.9	0.40	204
0	10	nothing	0.98	196	10.2	0.06	168
0	10	nothing	4.92	192	12.7	0.22	207
0	10	nothing	12.82	259	8.6	0.86	184
0	0	nothing	0.00	1390	10.6	0.00	143
0	0	nothing	11.46	264	14.2	0.67	199
0	0	nothing	7.13	1628	16.0	0.37	206
0	0	nothing	10.79	261	13.8	0.65	210
0	0	nothing	12.41	1704	14.5	0.71	200
0	0	nothing	13.24	185	13.8	0.80	192
10	10	adenine	0.00	1387	2.3	0.00	147
10	10	adenine	0.00	1227	6.8	0.00	117
10	10	adenine	2.94	285	5.6	0.21	157

[1]FOM = apparently fermented OM.

Table A-2 (cont'd)

Thiamine (mM)	Amprolium (mM)	Intracellular inhibitor	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM, µmoles/mg	NH_4^+, ppm
10	10	adenine	0.30	1530	6.7	0.02	179
10	10	adenine	4.13	267	5.7	0.28	160
10	10	adenine	2.00	216	5.4	0.15	140
10	0	adenine	2.59	183	6.7	0.19	202
10	0	adenine	4.36	202	7.3	0.30	168
10	0	adenine	9.73	280	7.1	0.68	166
10	0	adenine	3.00	1539	7.5	0.20	167
10	0	adenine	6.90	1641	7.2	0.48	147
10	0	adenine	7.05	252	7.9	0.45	169
0	10	adenine	3.11	249	7.0	0.22	159
0	10	adenine	8.77	242	8.6	0.52	186
0	10	adenine	0.00	1478	6.4	0.00	150
0	10	adenine	1.00	1642	9.4	0.05	190
0	10	adenine	6.15	1593	7.0	0.45	192
0	10	adenine	3.77	1582	8.2	0.23	170
0	0	adenine	4.19	1830	12.2	0.24	196
0	0	adenine	9.74	276	12.7	0.54	186
0	0	adenine	6.18	251	12.6	0.34	211
0	0	adenine	4.09	1660	9.5	0.30	191
0	0	adenine	4.72	233	11.1	0.30	171
0	0	adenine	3.87	225	10.4	0.26	195
10	10	adenosine	0.95	1650	12.8	0.03	275
10	10	adenosine	0.64	192	12.3	0.02	277
10	10	adenosine	0.98	197	13.2	0.03	252
10	10	adenosine	2.90	1656	12.7	0.08	246
10	10	adenosine	0.39	1565	13.1	0.01	303
10	10	adenosine	1.13	254	12.5	0.03	217

[1]FOM = apparently fermented OM.

Table A-2 (cont'd)

Thiamine (mM)	Amprolium (mM)	Intracellular inhibitor	CH_4, µmoles	CO_2, µmoles	FOM, %	CH_4/FOM, µmoles /mg	NH_4^+, ppm
10	0	adenosine	2.74	268	-	-	285
10	0	adenosine	3.04	181	15.4	0.09	275
0	10	adenosine	2.95	199	16.6	0.08	307
0	10	adenosine	1.42	1776	15.4	0.04	290
0	10	adenosine	1.97	284	16.4	0.05	278
0	10	adenosine	1.57	264	16.2	0.04	297
0	10	adenosine	2.67	303	16.8	0.07	310
0	10	adenosine	4.57	264	15.6	0.13	291
0	0	adenosine	5.33	195	19.7	0.16	331
0	0	adenosine	0.00	1633	22.7	0.00	295
0	0	adenosine	2.30	1613	21.7	0.06	263
0	0	adenosine	7.22	264	20.6	0.21	286
0	0	adenosine	2.03	1617	21.2	0.06	301
0	0	adenosine	5.38	227	20.5	0.16	292

[1]FOM = apparently fermented OM.

Table A-3 Unprocessed data for Chapter 2, Experiment 1: VFA production

Thiamine (mM)	Amprolium (mM)	Intracellular inhibitor	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
10	10	nothing	0.00	84.25	60.72	1.66	18.59	4.19	2.56	3.47
10	10	nothing	29.33	89.58	65.28	2.66	16.78	5.17	1.41	2.54
10	10	nothing	0.00	81.65	63.04	0.00	18.52	2.11	2.77	3.67
10	10	nothing	0.00	90.19	54.83	0.66	18.48	3.11	1.72	2.12
10	10	nothing	26.44	104.23	70.23	0.00	17.46	3.23	1.69	3.45
10	10	nothing	30.75	102.48	72.68	4.51	20.53	9.49	2.87	2.99
10	0	nothing	5.96	103.09	53.26	0.00	19.11	5.74	2.60	3.42
10	0	nothing	0.00	95.36	55.00	0.50	20.27	4.10	3.05	3.81
10	0	nothing	29.02	132.11	55.66	9.04	25.64	8.63	6.18	7.21
10	0	nothing	0.00	95.41	53.77	1.22	18.84	2.99	3.26	4.88
10	0	nothing	0.00	96.27	53.67	1.43	19.19	5.91	2.31	3.82
10	0	nothing	0.00	92.02	51.73	0.15	19.20	4.34	2.24	4.69
0	10	nothing	0.00	87.51	52.14	2.05	18.41	0.00	3.18	5.20
0	10	nothing	0.00	91.02	51.55	2.06	19.30	2.92	2.23	2.85
0	10	nothing	9.86	94.97	57.09	0.00	21.06	4.80	3.14	4.36
0	10	nothing	54.11	82.83	69.92	0.00	14.44	2.55	2.30	2.88
0	10	nothing	12.27	103.55	70.31	3.56	23.54	7.21	2.74	3.87
0	10	nothing	0.00	78.34	51.59	0.00	17.98	4.09	3.21	3.25
0	0	nothing	39.01	39.58	18.04	0.96	0.00	7.61	1.59	0.00
0	0	nothing	0.00	87.98	66.28	0.00	18.10	3.80	3.39	4.68
0	0	nothing	0.00	96.55	69.57	0.00	18.46	3.74	3.14	4.09
0	0	nothing	0.00	81.03	65.81	0.00	16.70	4.32	3.53	4.05
0	0	nothing	0.00	88.95	73.82	0.45	18.52	2.12	2.55	1.66
0	0	nothing	0.00	92.47	71.82	0.00	17.06	0.00	1.12	0.39
10	10	adenine	43.53	29.56	7.99	0.00	0.00	1.46	0.00	0.00
10	10	adenine	53.76	43.11	32.54	2.89	0.00	1.38	0.40	0.00
10	10	adenine	0.00	51.33	45.34	0.00	5.84	0.00	0.00	1.84
10	10	adenine	33.03	53.13	39.69	0.00	3.77	0.00	3.40	0.00
10	10	adenine	0.00	52.29	42.36	0.00	3.48	0.26	3.98	0.00

Table A-3 (cont'd)

Thiamine (mM)	Amprolium (mM)	Intracellular inhibitor	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
10	10	adenine	0.00	48.10	38.24	0.00	2.03	2.40	5.35	0.00
10	0	adenine	0.00	60.45	35.93	0.63	2.63	2.50	0.00	1.09
10	0	adenine	0.00	57.18	37.50	2.28	0.59	8.71	6.00	1.68
10	0	adenine	0.00	57.93	30.72	2.36	0.89	8.49	7.52	0.41
10	0	adenine	0.00	65.61	38.38	0.81	2.44	5.34	6.51	2.23
10	0	adenine	0.00	58.06	35.74	1.03	2.14	7.17	6.69	0.00
10	0	adenine	6.71	65.67	40.54	0.00	2.18	2.50	1.13	1.49
0	10	adenine	0.00	50.22	42.45	0.00	3.45	0.00	0.29	0.00
0	10	adenine	9.24	63.62	41.79	0.00	4.70	0.77	4.74	0.44
0	10	adenine	32.32	21.42	0.00	0.00	0.00	4.43	0.15	1.46
0	10	adenine	36.37	62.72	50.43	0.00	7.28	0.00	0.93	0.00
0	10	adenine	0.00	47.91	37.66	0.00	6.00	0.00	0.00	0.47
0	10	adenine	0.00	60.42	45.50	4.34	11.99	1.02	0.02	0.70
0	0	adenine	12.82	78.07	60.18	0.30	8.81	0.55	0.18	0.00
0	0	adenine	4.22	80.40	62.11	0.86	9.47	0.61	0.00	0.00
0	0	adenine	10.59	76.28	59.17	0.00	7.11	0.29	1.04	2.23
0	0	adenine	0.00	60.11	51.63	0.00	4.56	0.00	0.37	0.50
0	0	adenine	0.00	64.64	53.12	0.00	4.66	0.00	4.79	1.50
0	0	adenine	0.00	62.03	46.68	3.00	0.83	6.84	0.68	2.27
10	10	adenosine	0.00	132.28	186.72	5.29	12.06	22.92	5.23	0.55
10	10	adenosine	0.00	133.11	172.00	0.47	10.58	20.08	1.51	1.78
10	10	adenosine	0.00	134.48	176.94	1.08	10.91	24.89	14.84	0.00
10	10	adenosine	0.00	134.75	192.58	2.61	8.88	21.74	10.09	0.00
10	10	adenosine	0.00	140.43	193.31	0.00	6.39	16.01	10.81	4.87
10	10	adenosine	0.00	140.45	180.17	4.12	0.00	11.05	8.50	0.23
10	0	adenosine	16.85	121.16	167.23	18.67	23.82	45.42	25.98	11.14
10	0	adenosine	0.47	151.97	211.34	2.68	0.00	5.20	1.44	0.00
10	0	adenosine	0.00	151.48	204.14	0.00	2.37	0.30	4.95	0.01

Table A-3 (cont'd)

Thiamine (mM)	Amprolium (mM)	Intracellular inhibitor	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
10	0	adenosine	0.00	154.67	212.22	4.42	4.76	0.23	2.78	0.00
10	0	adenosine	18.12	165.64	220.30	2.38	5.70	2.51	0.00	0.00
0	10	adenosine	22.31	162.28	219.91	1.61	6.81	2.82	1.40	0.80
0	10	adenosine	8.41	149.01	191.11	0.00	3.93	0.00	0.54	1.20
0	10	adenosine	10.60	157.14	199.79	0.00	5.13	4.94	1.96	0.14
0	10	adenosine	10.05	154.43	201.74	0.00	4.24	0.00	3.51	0.43
0	10	adenosine	11.11	160.38	206.42	0.00	4.86	0.00	3.75	0.00
0	10	adenosine	14.03	151.54	196.63	0.00	4.04	0.00	1.79	0.00
0	0	adenosine	0.00	152.53	200.32	0.00	6.01	7.33	0.08	1.53
0	0	adenosine	15.34	148.22	181.27	2.73	0.25	0.00	3.34	0.00
0	0	adenosine	13.94	161.67	207.30	0.00	4.09	0.00	4.61	0.00
0	0	adenosine	9.74	152.94	200.49	0.00	3.28	0.00	5.55	0.74
0	0	adenosine	9.12	158.15	214.60	0.00	3.48	0.00	4.57	0.00
0	0	adenosine	4.08	155.94	207.19	0.00	3.70	0.00	0.51	0.18

Table A-4 Unprocessed data for Chapter 2, Experiment 2: gases, fermentation and ammonia

Exp.	Additive	Conc. (mM)	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM, µmoles /mg	NH_4^+, ppm
2	control	0	551	2382	53.2	1.872	395
2	control	0	541	2391	53.1	1.844	404
2	control	0	424	2082	51.5	1.491	382
2	control	0	535	2355	53.3	1.816	377
2	adenine	10	438	2272	53.5	1.481	547
2	adenine	10	449	2281	52.6	1.543	538
2	adenine	10	438	2159	51.4	1.543	535
2	adenine	10	490	2262	51.8	1.712	490
2	ribose	10	562	2486	68.1	1.492	361
2	ribose	10	730	2474	67.6	1.952	357
2	ribose	10	769	2482	67.1	2.072	324
2	ribose	10	613	2466	64.5	1.718	316
2	ad + rib	10	614	2458	64.8	1.712	541
2	ad + rib	10	592	2425	68.2	1.569	465
2	ad + rib	10	565	2429	62.4	1.636	479
2	ad + rib	10	569	2416	62.2	1.654	464
2	adenosine	10	580	2470	67.5	1.554	611
2	adenosine	10	523	2454	68.0	1.391	704
2	adenosine	10	636	2425	65.2	1.763	597
2	adenosine	10	635	2425	64.2	1.789	587

[1]FOM = apparently fermented OM.

Table A-5 Unprocessed data for Chapter 2, Experiment 2: VFA production

Exp.	Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
2	control	0	-3.97	2299.16	706.57	17.01	240.16	-1.91	39.26	17.43
2	control	0	1.14	2314.47	704.25	10.08	228.59	4.15	27.39	37.04
2	control	0	25.61	2242.11	691.80	8.58	204.80	-1.26	27.58	48.95
2	control	0	-5.97	2239.76	721.66	16.08	217.98	-6.59	36.05	68.24
2	adenine	10	-10.17	2314.07	636.63	35.51	223.19	-6.59	33.09	59.08
2	adenine	10	-10.17	2301.71	628.09	32.64	210.66	-6.59	33.81	52.97
2	adenine	10	-5.68	2240.22	629.09	32.39	199.86	-6.59	27.82	58.97
2	adenine	10	14.39	2269.11	637.68	32.57	197.57	-6.59	26.48	58.14
2	ribose	10	2.05	2867.20	831.19	28.18	344.04	-1.41	42.14	60.23
2	ribose	10	-10.17	2874.82	799.36	29.43	331.37	-6.59	44.36	64.95
2	ribose	10	-10.17	2850.01	841.46	23.77	308.57	-1.12	38.06	73.79
2	ribose	10	-10.17	2742.14	839.52	21.77	294.12	-6.59	32.73	63.82
2	ad + rib	10	-4.16	2750.62	814.00	40.21	302.76	-6.59	31.57	56.85
2	ad + rib	10	-10.17	2888.47	854.75	37.41	318.92	-6.59	32.76	68.80
2	ad + rib	10	-10.17	2649.46	808.01	31.66	277.58	-6.59	24.40	67.96
2	ad + rib	10	-10.17	2653.52	794.25	31.00	283.11	-6.59	24.92	61.79
2	adenosine	10	-10.17	2830.39	795.75	46.41	324.78	-1.39	44.75	74.52
2	adenosine	10	-4.79	2809.49	859.39	43.51	319.93	-6.59	45.66	78.73
2	adenosine	10	-10.17	2719.78	859.65	38.65	296.11	-3.99	32.66	70.07
2	adenosine	10	-6.41	2662.99	852.97	35.02	287.76	-2.98	33.72	77.05

Table A-6 Unprocessed data for Chapter 2, Experiment 3: gases, fermentation, pH and ammonia

Exp	Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM, µmoles /mg	pH	NH_4^+, ppm
3	control	0	0.20	298	684	55.8	2.888	7.21	315
3	control	0	0.69	310	680	58.6	2.854	7.36	290
3	control	0	0.44	317	691	58.1	2.969	7.28	322
3	control	0	0.40	323	529	53.8	3.259	7.39	301
3	oxythiamine	5	0.32	248	674	54.5	2.485	7.15	286
3	oxythiamine	5	0.55	244	711	52.3	2.535	7.14	269
3	oxythiamine	5	0.49	220	612	52.5	2.272	7.33	276
3	oxythiamine	5	0.49	257	592	53.5	2.611	7.07	285
3	oxythiamine	10	0.49	256	575	54.7	2.523	7.04	241
3	oxythiamine	10	0.52	228	703	51.1	2.426	7.17	249
3	oxythiamine	10	0.45	232	566	49.6	2.541	6.89	235
3	oxythiamine	10	0.50	232	728	50.5	2.480	7.14	267
3	amprolium	5	0.71	287	726	56.0	2.794	7.45	290
3	amprolium	5	0.66	286	683	53.6	2.895	7.13	289
3	amprolium	5	0.65	282	618	52.8	2.908	7.2	302
3	amprolium	5	0.36	292	669	43.1	3.675	7.04	290
3	amprolium	10	0.27	267	733	47.5	3.052	7.2	296
3	amprolium	10	0.67	291	691	49.2	3.195	7.19	293
3	amprolium	10	0.49	282	679	49.8	3.083	7.36	271
3	amprolium	10	0.51	269	716	46.5	3.133	6.99	269

[1]FOM = apparently fermented OM.

Table A-7 Unprocessed data for Chapter 2, Experiment 3: VFA production

Exp	Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
3	control	0	-3.73	798.67	299.70	-7.88	76.50	-9.26	7.85	11.22
3	control	0	-16.46	844.12	303.21	-7.38	77.35	-9.26	9.17	16.77
3	control	0	-16.46	833.68	301.20	-7.84	78.90	-9.26	8.02	12.64
3	control	0	-16.46	778.11	288.36	-7.61	64.63	-9.26	5.48	15.69
3	oxythiamine	5	-6.44	725.68	349.45	-8.28	64.54	-6.22	6.28	17.02
3	oxythiamine	5	-16.46	704.17	342.47	-8.66	59.91	-9.26	6.03	14.03
3	oxythiamine	5	1.24	715.67	368.33	-9.14	49.19	-9.26	2.56	14.29
3	oxythiamine	5	15.81	705.83	351.04	-6.31	61.47	-9.26	5.61	17.43
3	oxythiamine	10	15.47	726.87	363.76	-6.38	66.17	-9.26	5.08	14.99
3	oxythiamine	10	-8.33	690.72	344.85	-8.85	53.97	-9.26	1.34	14.91
3	oxythiamine	10	6.31	671.67	346.51	-7.63	49.32	-9.26	4.22	9.34
3	oxythiamine	10	-12.37	696.78	336.84	-11.73	56.91	-9.26	4.14	10.07
3	amprolium	5	-5.97	804.88	296.44	-9.68	71.55	-9.26	4.75	16.62
3	amprolium	5	-5.27	778.77	284.36	-9.61	63.13	-9.26	9.42	14.87
3	amprolium	5	-1.40	766.10	280.88	-8.55	63.84	-9.26	2.69	17.46
3	amprolium	5	11.03	626.39	258.74	-5.13	46.58	-9.26	-0.44	7.49
3	amprolium	10	-3.08	714.25	244.14	-8.82	56.64	-9.26	2.06	10.37
3	amprolium	10	9.14	737.87	255.96	-8.91	57.32	-9.26	4.42	12.48
3	amprolium	10	35.69	737.87	255.05	-8.19	59.12	-9.26	5.69	10.76
3	amprolium	10	-0.37	704.94	250.51	-8.61	49.25	-9.26	2.48	8.65

Table A-8 Unprocessed data for Chapter 2, Experiment 4: gases, fermentation, pH and ammonia

Exp	Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM, µmoles /mg	pH	NH_4^+, ppm
4	control	0	0.51	448	963	58.2	2.772	6.73	140
4	control	0	0.48	462	965	57.4	2.924	6.76	166
4	control	0	0.47	479	1132	57.2	3.030	6.82	139
4	control	0	0.04	467	1104	58.3	2.888	6.72	158
4	pyruvate	2	0.42	468	1124	61.9	2.723	6.84	164
4	pyruvate	2	0.40	483	1105	61.3	2.847	6.87	169
4	pyruvate	2	0.35	447	1069	58.7	2.758	6.79	168
4	pyruvate	2	0.35	418	917	60.8	2.486	6.73	175
4	Br-pyruvate	2	0.36	440	1179	56.4	2.818	6.71	162
4	Br-pyruvate	2	0.25	337	543	57.1	2.132	6.78	166
4	Br-pyruvate	2	0.28	434	1156	59.7	2.636	6.85	160
4	Br-pyruvate	2	0.44	467	1193	60.1	2.809	6.73	158
4	F-pyruvate	2	0.73	397	1002	59.6	2.416	6.67	162
4	F-pyruvate	2	0.69	446	1158	60.5	2.660	6.81	172
4	F-pyruvate	2	0.81	419	1112	54.2	2.806	6.8	163
4	F-pyruvate	2	1.00	418	1088	54.4	2.784	6.86	145
4	HO-pyruvate	2	0.35	425	1049	65.8	2.325	6.72	147
4	HO-pyruvate	2	0.44	459	1085	66.5	2.488	6.74	153
4	HO-pyruvate	2	0.28	449	1100	65.7	2.460	6.76	157
4	HO-pyruvate	2	0.48	466	1087	67.0	2.506	6.73	165

[1]FOM = apparently fermented OM.

Table A-9 Unprocessed data for Chapter 2, Experiment 4: VFA production

Exp	Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
4	control	0	-3.65	1257	337	5.86	139	0.00	28.7	27.3
4	control	0	-3.65	1224	324	6.01	139	0.00	29.2	27.5
4	control	0	-3.65	1234	342	5.86	126	0.00	28.3	28.1
4	control	0	1.47	1264	339	6.77	139	0.00	27.4	24.4
4	pyruvate	2	-3.65	1352	371	7.67	135	0.00	30.3	26.3
4	pyruvate	2	1.25	1344	347	7.15	139	0.00	31.3	23.7
4	pyruvate	2	-3.65	1286	338	6.52	129	0.00	27.8	24.7
4	pyruvate	2	2.45	1334	344	6.04	136	0.00	29.8	26.6
4	Br-pyruvate	2	6.30	1209	343	7.42	120	0.00	34.0	26.8
4	Br-pyruvate	2	-3.65	1246	329	5.88	131	0.00	24.3	28.2
4	Br-pyruvate	2	-3.65	1280	329	6.69	148	0.00	30.8	27.1
4	Br-pyruvate	2	0.84	1303	335	6.40	142	0.00	30.1	28.8
4	F-pyruvate	2	1.52	1292	353	6.37	135	0.00	24.8	25.7
4	F-pyruvate	2	-0.35	1304	330	6.95	154	0.00	30.9	27.3
4	F-pyruvate	2	0.25	1194	334	4.77	108	0.00	19.8	26.3
4	F-pyruvate	2	-0.95	1204	334	4.91	113	0.00	19.6	20.4
4	HO-pyruvate	2	-3.65	1321	371	13.00	199	0.00	37.7	32.1
4	HO-pyruvate	2	5.62	1347	386	14.41	191	0.00	36.0	30.4
4	HO-pyruvate	2	6.80	1340	399	12.41	182	0.00	32.7	29.6
4	HO-pyruvate	2	0.93	1337	373	14.34	206	0.00	39.9	33.2

Table A-10 Unprocessed data for Chapter 3, Experiment 1, Run 1: gases, fermentation, pH and ammonia

Run	Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	Fermented substrate, %	CH_4/FOM, µmoles/mg	pH	NH_4^+, ppm
1	control	0	0.53	395	718	54.7	54.7	2.614	6.91	266
1	control	0	0.55	427	882	58.7	58.7	2.628	6.83	267
1	oxalacetate	6	0.53	473	1096	57.8	51.8	2.567	7.02	291
1	oxalacetate	6	0.65	456	906	53.2	46.4	2.690	6.95	239
1	acetoacetate	6		411	807	56.2	51.3	2.393	7.08	276
1	acetoacetate	6	0.50	391	979	60.9	56.5	2.101	6.81	262
1	B-hydroxybutyrate	6	0.46	435	890	59.1	59.0	2.384	6.91	277
1	B-hydroxybutyrate	6	0.50	394	922	52.7	52.0	2.425	6.97	243
1	Crotonate	6	0.68	411	927	56.7	56.7	2.386	7.19	261
1	Crotonate	6	0.45	403	922	54.6	54.9	2.432	6.94	253
1	Oxaloacetate	12	-	-	-	38.2	20.2	-	6.72	245
1	Oxaloacetate	12	0.57	456	1082	55.6	42.6	2.295	6.92	257
1	Acetoacetate	12	0.46	267	468	55.9	47.5	1.409	6.94	252
1	Acetoacetate	12	0.40	367	1001	61.8	55.0	1.747	6.87	210
1	B-hydroxybutyrate	12	0.75	439	840	51.5	52.2	2.516	6.82	258
1	B-hydroxybutyrate	12	0.57	407	824	51.7	54.3	2.328	7	257
1	Crotonate	12	0.54	384	994	57.7	56.7	2.025	6.83	261
1	Crotonate	12	0.66	372	946	58.1	57.0	1.941	6.69	240
1	Oxaloacetate	18	0.65	480	1251	51.7	30.4	2.342	6.92	237
1	Oxaloacetate	18	0.89	498	1357	56.7	37.7	2.208	6.87	274
1	Acetoacetate	18	0.33	374	1007	58.2	53.3	1.746	7.04	264
1	Acetoacetate	18	0.35	424	1033	65.6	59.3	1.740	7.03	291
1	B-hydroxybutyrate	18	0.50	427	962	53.7	60.9	2.152	7.21	235
1	B-hydroxybutyrate	18	0.58	482	1103	60.1	65.2	2.152	7	304
1	Crotonate	18	0.66	393	1026	60.2	58.0	1.834	6.82	218
1	Crotonate	18	0.48	447	1009	64.0	60.7	1.970	6.75	293

[1]FOM = apparently fermented OM.

Table A-11 Unprocessed data for Chapter 3, Experiment 1, Run 1: VFA production

Run	Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
1	Control	0	-1.95	1071	327	31.51	137	0.00	33.8	23.8
1	Control	0	-1.95	1104	347	33.40	138	0.00	32.4	24.7
1	Oxaloacetate	6	-1.95	1276	345	30.33	151	0.00	34.1	24.3
1	Oxaloacetate	6	20.64	1233	344	18.04	134	0.00	33.3	26.2
1	Acetoacetate	6	2.31	1383	299	2.55	161	0.00	34.9	23.0
1	Acetoacetate	6	-1.95	1460	318	30.80	160	0.00	30.5	17.7
1	B-hydroxybutyrate	6	923.28	1303	339	37.74	220	0.00	33.1	24.7
1	B-hydroxybutyrate	6	953.04	1142	313	2.95	176	0.00	22.5	21.0
1	Crotonate	6	15.23	1181	327	8.03	250	0.00	-	28.3
1	Crotonate	6	9.51	1135	322	4.49	229	0.00	-	24.4
1	Oxaloacetate	12	1.43	1430	339	31.25	153	0.00	33.4	25.8
1	Oxaloacetate	12	6.93	1513	365	33.08	152	0.00	29.4	24.8
1	Acetoacetate	12	76.53	1847	316	6.96	207	0.00	46.4	27.0
1	Acetoacetate	12	96.46	1811	315	11.35	202	0.00	37.8	22.5
1	B-hydroxybutyrate	12	2246.49	1290	303	6.89	243	0.00	26.5	20.7
1	B-hydroxybutyrate	12	2602.78	1309	318	4.46	233	0.00	26.7	23.5
1	Crotonate	12	42.62	1365	308	0.78	322	0.00	-	23.0
1	Crotonate	12	59.54	1397	318	-2.41	343	0.00	-	29.4
1	Oxalacetate	18	-1.95	1578	339	-1.35	151	0.00	30.9	26.4
1	Oxalacetate	18	15.42	1695	362	10.69	188	0.00	43.6	31.7
1	Acetoacetate	18	505.14	1792	310	12.02	245	0.00	45.2	20.3
1	Acetoacetate	18	287.79	2122	321	32.38	274	0.00	58.4	25.0
1	B-hydroxybutyrate	18	4356.02	1467	337	33.23	280	0.00	28.5	22.6
1	B-hydroxybutyrate	18	3562.49	1645	340	14.21	346	0.00	42.0	28.4
1	Crotonate	18	89.99	1609	320	4.94	410	0.00	-	27.7
1	Crotonate	18	87.69	1714	306	9.98	465	0.00	-	30.9

Table A-12 Unprocessed data for Chapter 3, Experiment 1, Run 2: gases, fermentation, pH and ammonia

Run	Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	Fermented substrate, %	CH_4/FOM µmoles/mg	pH	NH_4^+, ppm
2	Control	0	0.28	435	1030	59.0	59.0	2.667	6.83	260
2	Control	0	0.28	425	1014	59.8	59.8	2.573	6.88	254
2	Oxaloacetate	6	0.31	442	1172	62.6	57.1	2.219	6.7	227
2	Oxaloacetate	6	0.34	436	1191	59.3	53.9	2.319	7.27	240
2	Acetoacetate	6	0.25	399	1054	65.9	62.2	1.967	6.86	218
2	Acetoacetate	6	0.30	399	1109	66.7	63.3	1.932	6.76	235
2	B-hydroxybutyrate	6	0.25	423	878	59.2	60.6	2.314	6.72	223
2	B-hydroxybutyrate	6	0.35	447	1019	60.5	61.8	2.384	6.77	252
2	Crotonate	6	0.30	398	1050	64.5	67.2	2.026	6.75	245
2	Crotonate	6	0.28	411	1154	64.4	67.3	2.115	6.89	251
2	Oxaloacetate	12	0.37	454	1427	61.1	50.9	2.082	6.77	231
2	Oxaloacetate	12	0.40	451	1321	62.2	51.1	2.033	6.75	203
2	Acetoacetate	12	0.29	376	1123	69.0	63.1	1.610	6.78	243
2	Acetoacetate	12	0.27	374	1152	66.5	60.9	1.660	6.75	245
2	B-hydroxybutyrate	12	0.32	423	1028	57.1	62.2	2.183	6.77	247
2	B-hydroxybutyrate	12	0.36	422	1057	58.8	65.0	2.118	6.81	255
2	Crotonate	12	0.55	407	1113	58.4	57.5	2.116	6.7	229
2	Crotonate	12	0.52	376	974	56.0	54.5	2.044	6.66	267
2	Oxaloacetate	18	0.48	462	1316	55.3	37.0	2.095	7.06	252
2	Oxaloacetate	18	0.40	464	1383	57.0	38.1	2.048	6.91	246
2	Acetoacetate	18	0.21	370	1089	66.6	62.7	1.501	6.87	237
2	Acetoacetate	18	0.23	341	865	64.7	60.1	1.432	6.68	232
2	B-hydroxybutyrate	18	0.32	423	1034	51.3	61.3	2.224	6.74	204
2	B-hydroxybutyrate	18	0.31	435	1065	54.8	66.2	2.128	6.76	247
2	Crotonate	18	0.39	408	1106	67.7	70.4	1.699	6.61	242
2	Crotonate	18	0.30	403	1365	76.9	83.7	1.471	6.51	243

[1]FOM = apparently fermented OM.

Table A-13 Unprocessed data for Chapter 3, Experiment 1, Run 2: VFA production

Run	Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
2	Control	0	-13.14	1132	344	0.21	132	0.00	20.8	14.3
2	Control	0	-12.40	1138	360	2.50	137	0.00	24.3	18.9
2	Oxaloacetate	6	-7.87	1451	400	23.00	168	0.00	30.0	22.7
2	Oxaloacetate	6	-9.94	1361	373	-3.12	149	0.00	25.3	20.0
2	Acetoacetate	6	-8.41	1668	364	6.89	182	0.00	29.7	19.4
2	Acetoacetate	6	-2.51	1658	364	11.03	187	0.00	34.0	20.6
2	B-hydroxybutyrate	6	1166.79	1382	375	8.90	219	0.00	28.6	22.7
2	B-hydroxybutyrate	6	1138.87	1355	367	8.61	213	0.00	28.7	24.0
2	crotonate	6	3.30	1403	387	27.26	274	0.00	232.3	22.6
2	crotonate	6	14.73	1357	366	4.99	269	0.00	238.4	23.7
2	Oxaloacetate	12	-5.63	1592	394	6.67	172	0.00	32.4	25.4
2	Oxaloacetate	12	-1.38	1653	409	36.10	183	0.00	36.3	29.6
2	Acetoacetate	12	39.70	2077	349	4.54	213	0.00	41.3	23.6
2	acetoacetate	12	89.25	1954	339	-0.69	211	0.00	36.5	21.6
2	B-hydroxybutyrate	12	2827.76	1427	365	2.76	245	0.00	28.9	25.3
2	B-hydroxybutyrate	12	2972.37	1489	364	26.17	248	0.00	21.4	14.7
2	Crotonate	12	11.24	1368	358	2.06	276	0.00	262.1	21.8
2	Crotonate	12	13.45	1343	355	2.81	266	0.00	257.0	21.2
2	oxalacetate	18	1.40	1725	391	1.69	172	0.00	29.6	24.4
2	oxalacetate	18	5.72	1753	395	10.02	176	0.00	30.3	23.9
2	acetoacetate	18	386.71	2245	360	9.52	258	0.00	38.8	16.9
2	Acetoacetate	18	388.66	2280	358	15.79	260	0.00	37.9	17.6
2	B-hydroxybutyrate	18	5036.95	1415	356	-1.13	236	0.00	25.1	21.3
2	B-hydroxybutyrate	18	5084.30	1516	380	14.59	261	0.00	28.8	24.0
2	Crotonate	18	104.01	1929	343	1.02	448	0.00	438.1	25.2
2	Crotonate	18	129.94	2164	380	13.64	500	0.00	493.6	25.2

Table A-14 Unprocessed data for Chapter 3, Experiment 2, Run 1: gases, fermentation, pH and ammonia

Run	Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	Fermented substrate, %	CH_4/FOM µmoles/mg	pH	NH_4^+, ppm
1	control	0	1.17	489	1904	63.0	63.0	2.788	7.01	275
1	control	0	1.13	461	1708	64.0	64.0	2.592	7.11	275
1	propynoic acid	6	47.56	163	1660	55.3	59.9	0.984	7.04	206
1	propynoic acid	6	-	159	1488	63.2	68.6	0.841	7.05	193
1	3-butenoic acid	6	1.70	486	1692	77.9	75.8	2.050	6.79	224
1	3-butenoic acid	6	0.22	476	2009	82.4	80.8	1.898	6.73	267
1	2-butynoic acid	6	0.65	467	1349	68.4	74.0	2.249	6.92	275
1	2-butynoic acid	6	0.96	456	1506	65.7	71.1	2.287	7	269
1	ethyl 2-butynoate	6	0.85	492	1631	68.5	64.6	2.317	6.75	242
1	ethyl 2-butynoate	6	1.80	413	1596	69.8	66.0	1.896	6.93	220
1	propynoic acid	12	-	126	1178	57.1	66.7	0.688	6.73	218
1	propynoic acid	12	-	127	1591	56.7	66.4	0.704	6.89	187
1	3-butenoic acid	12	1.09	452	1613	74.1	69.1	1.856	6.84	251
1	3-butenoic acid	12	0.56	407	1163	69.2	63.4	1.785	6.64	248
1	2-butynoic acid	12	0.79	473	1866	48.9	56.7	2.955	6.83	252
1	2-butynoic acid	12	1.85	452	1915	48.3	56.7	2.845	6.87	219
1	ethyl 2-butynoate	12	13.17	399	1796	63.0	53.9	1.829	6.88	183
1	ethyl 2-butynoate	12	34.71	148	1161	61.9	52.5	0.693	6.8	199
1	propynoic acid	18	-	113	1211	56.4	70.8	0.592	6.58	176
1	propynoic acid	18	-	102	677	58.5	72.9	0.511	6.71	179
1	3-butenoic acid	18	7.16	449	1742	89.5	86.6	1.406	6.62	257
1	3-butenoic acid	18	0.82	396	1535	86.3	82.4	1.283	6.79	231
1	2-butynoic acid	18	0.93	454	1582	54.0	66.1	2.367	6.63	247
1	2-butynoic acid	18	1.89	429	1987	50.0	62.1	2.418	6.81	238
1	ethyl 2-butynoate	18	35.84	235	1527	52.3	34.5	1.184	6.74	178
1	ethyl 2-butynoate	18	-	54	1257	49.0	29.9	0.293	6.94	179

[1]FOM = apparently fermented OM.

Table A-15 Unprocessed data for Chapter 3, Experiment 2, Run 1: VFA production

Run	Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
1	Control	0	5.28	1400	390	41.44	200	4.15	54.8	39.9
1	Control	0	49.58	1383	361	19.18	175	2.66	52.7	41.5
1	propynoic acid	6	177.53	1091	422	6.52	203	85.61	38.1	51.0
1	propynoic acid	6	174.01	1166	454	17.92	228	113.78	58.5	73.7
1	3-butenoic acid	6	113.92	1738	-	30.32	379	28.42	106.1	104.5
1	3-butenoic acid	6	173.51	1746	-	46.77	401	72.74	117.9	120.6
1	2-butynoic acid	6	44.99	1403	380	-	259	39.10	127.4	157.0
1	2-butynoic acid	6	34.12	1365	366	-	246	35.30	105.7	145.4
1	ethyl 2-butynoate	6	88.06	1461	441	73.40	-	104.35	97.2	112.9
1	ethyl 2-butynoate	6	190.48	1429	447	136.35	-	221.74	92.9	110.7
1	propynoic acid	12	328.33	1052	371	46.02	218	151.38	63.5	45.5
1	propynoic acid	12	311.40	1054	381	40.97	199	204.80	52.6	24.5
1	3-butenoic acid	12	180.30	2141	-	41.59	518	24.70	113.9	96.0
1	3-butenoic acid	12	214.37	2024	-	36.51	477	16.76	96.5	75.3
1	2-butynoic acid	12	27.56	1337	352	-	218	20.92	89.5	124.0
1	2-butynoic acid	12	25.50	1313	353	-	224	30.94	102.0	122.1
1	ethyl 2-butynoate	12	77.06	1352	460	167.60	-	251.64	69.6	83.0
1	ethyl 2-butynoate	12	331.28	1055	427	299.81	-	474.72	66.2	76.2
1	propynoic acid	18	287.80	1012	339	38.03	197	136.26	43.8	53.1
1	propynoic acid	18	334.37	995	341	46.61	196	144.63	54.4	93.5
1	3-butenoic acid	18	232.07	2413	-	44.13	612	20.73	129.6	79.9
1	3-butenoic acid	18	220.17	2412	-	52.34	606	5.71	101.6	61.0
1	2-butynoic acid	18	58.14	1261	319	-	210	8.69	62.7	83.9
1	2-butynoic acid	18	4.73	1267	316	-	192	7.60	50.1	64.1
1	ethyl 2-butynoate	18	210.56	1054	410	244.04	-	339.19	26.5	26.9
1	ethyl 2-butynoate	18	453.69	859	349	492.14	-	658.42	54.4	45.7

Table A-16 Unprocessed data for Chapter 3, Experiment 2, Run 2: gases, fermentation, pH and ammonia

Run	Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	Fermented substrate, %	CH_4/FOM µmoles/mg	pH	NH_4^+, ppm
2	control	0	1.17	489	1904	63.0	63.0	2.788	7.01	275
2	control	0	1.13	461	1708	64.0	64.0	2.592	7.11	275
2	propynoic acid	6	47.56	163	1660	55.3	59.9	0.984	7.04	206
2	propynoic acid	6	-	159	1488	63.2	68.6	0.841	7.05	193
2	3-butenoic acid	6	1.70	486	1692	77.9	75.8	2.050	6.79	224
2	3-butenoic acid	6	0.22	476	2009	82.4	80.8	1.898	6.73	267
2	2-butynoic acid	6	0.65	467	1349	68.4	74.0	2.249	6.92	275
2	2-butynoic acid	6	0.96	456	1506	65.7	71.1	2.287	7.00	269
2	ethyl 2-butynoate	6	0.85	492	1631	68.5	64.6	2.317	6.75	242
2	ethyl 2-butynoate	6	1.80	413	1596	69.8	66.0	1.896	6.93	220
2	propynoic acid	12	-	126	1178	57.1	66.7	0.688	6.73	218
2	propynoic acid	12	-	127	1591	56.7	66.4	0.704	6.89	187
2	3-butenoic acid	12	1.09	452	1613	74.1	69.1	1.856	6.84	251
2	3-butenoic acid	12	0.56	407	1163	69.2	63.4	1.785	6.64	248
2	2-butynoic acid	12	0.79	473	1866	48.9	56.7	2.955	6.83	252
2	2-butynoic acid	12	1.85	452	1915	48.3	56.7	2.845	6.87	219
2	ethyl 2-butynoate	12	13.17	399	1796	63.0	53.9	1.829	6.88	183
2	ethyl 2-butynoate	12	34.71	148	1161	61.9	52.5	0.693	6.8	199
2	propynoic acid	18	-	113	1211	56.4	70.8	0.592	6.58	176
2	propynoic acid	18	-	102	677	58.5	72.9	0.511	6.71	179
2	3-butenoic acid	18	7.16	449	1742	89.5	86.6	1.406	6.62	257
2	3-butenoic acid	18	0.82	396	1535	86.3	82.4	1.283	6.79	231
2	2-butynoic acid	18	0.93	454	1582	54.0	66.1	2.367	6.63	247
2	2-butynoic acid	18	1.89	429	1987	50.0	62.1	2.418	6.81	238
2	ethyl 2-butynoate	18	35.84	235	1527	52.3	34.5	1.184	6.74	178
2	ethyl 2-butynoate	18	-	54	1257	49.0	29.9	0.293	6.94	179

[1]FOM = apparently fermented OM.

Table A-17 Unprocessed data for Chapter 3, Experiment 2, Run 2: VFA production

Run	Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
2	Control	0	-12.23	1060	313	6.23	104	-7.36	12.7	5.8
2	Control	0	1.44	1054	305	7.90	97	-7.36	9.6	2.8
2	propynoic acid	6	97.34	829	362	7.24	98	25.74	-4.5	-13.5
2	propynoic acid	6	40.46	756	327	-1.26	78	21.18	-9.0	-14.4
2	3-butenoic acid	6	121.14	1397	-	29.98	283	26.94	80.6	66.9
2	3-butenoic acid	6	110.21	1431	-	24.68	297	24.51	73.0	72.0
2	2-butynoic acid	6	29.55	1061	334	-	157	26.05	61.0	115.6
2	2-butynoic acid	6	19.51	1114	334	-	168	24.82	65.7	99.7
2	ethyl 2-butynoate	6	237.30	899	414	137.96	-	406.30	47.4	58.6
2	ethyl 2-butynoate	6	197.55	937	406	144.55	-	390.47	53.7	68.3
2	propynoic acid	12	252.14	787	-	5.74	101	152.31	28.1	12.6
2	propynoic acid	12	236.37	795	-	7.88	101	129.80	32.4	3.4
2	3-butenoic acid	12	99.61	1735	326	19.31	381	4.22	58.0	36.7
2	3-butenoic acid	12	138.56	1794	327	18.84	396	4.05	63.2	34.8
2	2-butynoic acid	12	0.29	951	268	-	110	-3.02	17.9	29.1
2	2-butynoic acid	12	-12.23	975	272	-	108	-7.36	12.9	22.5
2	ethyl 2-butynoate	12	265.33	684	285	258.43	-	493.10	59.9	19.8
2	ethyl 2-butynoate	12	263.77	634	262	256.34	-	471.61	42.9	11.1
2	propynoic acid	18	215.82	760	-	2.07	73	72.45	2.5	-3.8
2	propynoic acid	18	197.13	752	-	4.15	73	75.10	1.9	-6.2
2	3-butenoic acid	18	152.97	1994	317	10.40	476	6.74	26.6	37.8
2	3-butenoic acid	18	210.22	2085	343	22.17	508	14.71	22.9	54.6
2	2-butynoic acid	18	41.20	976	276	-	124	7.15	34.1	38.6
2	2-butynoic acid	18	5.87	995	275	-	121	13.03	28.8	49.2
2	ethyl 2-butynoate	18	271.47	609	275	301.96	-	584.42	74.6	32.9
2	ethyl 2-butynoate	18	302.31	647	283	322.35	-	592.08	68.4	24.3

Table A-18 Unprocessed data for Chapter 4, aphidicolin: gases, fermentation, pH and ammonia

Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM µmoles/mg	pH	NH_4^+, ppm
Aphidicolin	0	0.10	34	982	41.6	0.444	6.93	32
Aphidicolin	0	0.07	38	898	50.7	0.405	6.85	34
Aphidicolin	0	0.00	20	226	36.7	0.288	-	35
Aphidicolin	0	0.03	50	-	41.1	0.661	7.24	34
Aphidicolin	0	0.11	119	741	40.8	1.588	7.17	33
Aphidicolin	30	0.10	17	-	43.1	0.210	6.88	98
Aphidicolin	30	0.17	66	843	44.1	0.812	6.88	107
Aphidicolin	30	0.10	33	504	43.2	0.414	6.85	59
Aphidicolin	30	0.07	51	199	38.9	0.717	7.09	106
Aphidicolin	30	0.14	12	94	37.6	0.177	7.41	103
Aphidicolin	60	0.13	37	863	45.6	0.436	6.88	64
Aphidicolin	60	0.09	34	760	38.8	0.473	6.84	57
Aphidicolin	60	0.07	80	387	45.3	0.960	6.88	57
Aphidicolin	60	0.07	39	103	36.8	0.579	7.47	55
Aphidicolin	60	0.00	85	886	38.0	1.202	7.08	62
Aphidicolin	120	0.06	34	879	39.1	0.478	6.94	33
Aphidicolin	120	0.28	39	812	41.8	0.505	6.88	34
Aphidicolin	120	0.00	59	1008	37.7	0.857	6.81	21
Aphidicolin	120	0.32	88	927	37.7	1.268	6.82	30
Aphidicolin	120	0.08	70	750	39.6	0.960	6.95	31

[1]FOM = apparently fermented OM.

Table A-19 Unprocessed data for aphidicolin: VFA production

Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Aphidicolin	0	0.24	560	178	36.31	105	-6.12	8.0	-0.6
Aphidicolin	0	-2.02	645	288	56.06	112	-6.28	9.3	10.7
Aphidicolin	0	-2.61	527	174	16.38	68	-6.24	8.4	-0.6
Aphidicolin	0	0.19	567	180	26.83	92	-6.27	9.4	8.3
Aphidicolin	0	-0.51	480	226	50.35	109	0.34	6.4	8.3
Aphidicolin	30	-1.69	517	235	66.08	114	-6.30	6.9	9.7
Aphidicolin	30	-0.30	534	204	42.69	135	25.76	21.1	-0.8
Aphidicolin	30	-2.26	549	211	65.32	109	-6.41	8.2	9.3
Aphidicolin	30	-2.39	518	172	33.46	97	-6.26	7.6	9.7
Aphidicolin	30	-1.78	534	174	15.44	73	-6.15	8.1	10.7
Aphidicolin	60	17.51	590	251	53.91	99	-1.58	7.4	9.2
Aphidicolin	60	-3.45	526	165	17.87	95	-6.19	8.6	9.6
Aphidicolin	60	-2.10	526	168	30.31	168	51.16	75.7	14.5
Aphidicolin	60	-1.76	497	190	47.28	74	-6.52	6.3	9.9
Aphidicolin	60	-1.76	527	174	17.85	84	-3.72	7.7	8.5
Aphidicolin	120	-2.21	552	180	14.66	76	-6.24	6.5	-0.6
Aphidicolin	120	-0.77	576	217	25.34	81	-1.91	8.6	-0.6
Aphidicolin	120	-2.24	563	177	7.98	58	-6.53	7.2	-0.8
Aphidicolin	120	-2.17	524	196	37.33	68	-6.32	6.1	7.7
Aphidicolin	120	-2.06	570	176	15.51	77	-6.48	8.1	-0.6

Table A-20 Unprocessed data for Chapter 4, lumazine, BPS and BES: gases, fermentation, pH and ammonia

Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM, µmoles/mg	pH	NH_4^+, ppm
Control	0	0.28	93	709	44.8	1.121	6.92	169
Control	0	0.23	100	942	42.3	1.284	6.93	177
Control	0	0.26	68	499	44.8	0.822	6.81	187
Control	0	0.35	140	878	43.3	1.750	6.9	165
Control	0	0.32	142	704	42.6	1.819	7.05	178
Lumazine	0.3	0.34	122	588	36.4	1.815	7.03	175
Lumazine	0.3	0.27	112	339	41.7	1.463	6.94	174
Lumazine	0.3	-	-	-	39.8	0.000	6.85	181
Lumazine	0.3	0.30	98	471	36.7	1.452	6.97	166
Lumazine	0.3	0.48	146	847	37.8	2.082	6.99	172
Lumazine	0.6	0.18	56	679	38.3	0.789	6.94	179
Lumazine	0.6	0.34	93	523	35.5	1.416	7.13	189
Lumazine	0.6	0.00	35	320	36.5	0.526	6.9	176
Lumazine	0.6	0.15	37	88	34.4	0.581	7.1	162
Lumazine	0.6	0.10	49	-	35.6	0.748	7.22	176
Lumazine	1.2	0.15	54	394	37.3	0.788	-	163
Lumazine	1.2	0.35	66	772	35.7	1.004	6.88	178
Lumazine	1.2	0.53	70	470	37.0	1.026	6.91	179
Lumazine	1.2	0.16	63	477	36.6	0.938	7.02	143
Lumazine	1.2	0.12	28	169	34.0	0.451	7.13	175
BPS	1	0.25	62	664	36.9	0.911	-	168
BPS	1	0.39	86	843	37.2	1.259	6.92	192
BPS	1	0.15	49	-	38.2	0.694	7.03	195
BPS	1	0.22	83	192	37.9	1.187	7.1	181
BPS	1	0.21	96	542	35.8	1.453	6.99	172
BPS	10	0.22	86	968	37.0	1.267	-	182
BPS	10	0.66	90	982	41.3	1.186	6.91	187
BPS	10	0.43	125	972	39.8	1.709	6.75	176

[1]FOM = apparently fermented OM.

Table A-20 (cont'd)

Additive	Conc. (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM µmoles /mg	pH	NH_4^+, ppm
BPS	10	0.30	127	475	37.7	1.829	6.93	177
BPS	10	0.02	20	73	37.4	0.292	-	169
BPS	50	0.17	77	477	38.7	1.082	6.97	186
BPS	50	0.64	87	724	36.0	1.304	6.93	180
BPS	50	0.15	88	830	40.2	1.192	6.79	185
BPS	50	0.22	120	541	35.2	1.850	7.12	166
BPS	50	0.47	141	837	38.1	2.000	-	180
BES	1	2.25	50	560	39.8	0.685	7.07	183
BES	1	1.01	53	771	39.8	0.725	6.84	151
BES	1	1.30	54	612	40.8	0.717	6.75	175
BES	1	0.99	90	379	37.5	1.295	7.05	173
BES	1	1.56	33	294	36.4	0.498	-	177
BES	10	6.70	50	439	41.7	0.653	6.97	186
BES	10	6.52	32	749	39.0	0.440	6.81	167
BES	10	0.03	4	56	38.3	0.059	7.31	162
BES	10	8.30	43	818	39.6	0.587	7.02	157
BES	10	3.11	18	149	38.8	0.253	-	154
BES	50	5.84	22	523	40.1	0.298	7.03	174
BES	50	4.98	38	171	40.5	0.512	6.87	185
BES	50	5.34	22	326	37.8	0.313	7.11	143
BES	50	3.83	17	219	40.4	0.235	6.88	148
BES	50	9.46	35	756	38.3	0.496	-	157

[1]FOM = apparently fermented OM.

Table A-21 Unprocessed data for Chapter 4, lumazine, BPS and BES: VFA production

Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Control	0	-0.42	528	174	37.33	161	34.95	16.4	12.6
Control	0	0.54	530	175	33.68	130	-0.60	8.9	14.6
Control	0	-2.11	556	236	72.26	114	-6.34	9.8	14.8
Control	0	-2.43	532	245	45.34	105	-6.33	8.3	11.3
Control	0	-2.08	487	158	36.57	161	41.21	14.6	13.5
Lumazine	0.3	-2.20	536	184	1.21	54	-6.19	8.3	13.2
Lumazine	0.3	-1.81	609	204	11.63	68	-5.47	11.0	14.1
Lumazine	0.3	-1.04	575	192	7.52	71	-0.24	11.5	12.5
Lumazine	0.3	-2.58	509	190	10.54	68	10.17	10.2	10.3
Lumazine	0.3	-2.16	518	198	20.76	74	-6.47	7.8	14.0
Lumazine	0.6	1.13	561	191	4.68	59	-6.25	9.2	13.6
Lumazine	0.6	-2.42	522	167	7.97	60	-3.53	8.9	13.5
Lumazine	0.6	-2.43	546	175	3.74	55	-6.29	8.6	11.8
Lumazine	0.6	-0.31	516	175	4.62	45	-6.38	7.0	11.2
Lumazine	0.6	-1.39	546	174	0.01	47	-6.37	8.9	12.4
Lumazine	1.2	-2.23	555	181	55.61	58	-6.19	9.0	13.3
Lumazine	1.2	-0.94	537	170	28.30	52	-6.42	9.1	12.6
Lumazine	1.2	-2.35	548	174	56.08	59	-6.02	8.0	13.3
Lumazine	1.2	-2.36	550	184	42.80	49	-6.29	7.1	11.0
Lumazine	1.2	-2.61	510	169	27.97	49	-6.24	8.1	12.1
BPS	1	-2.08	545	179	-0.32	56	-6.40	8.1	12.7
BPS	1	-2.17	539	172	5.43	66	-6.43	9.4	13.7
BPS	1	0.55	553	179	10.26	70	-5.86	10.8	14.5
BPS	1	-2.28	555	182	4.38	62	-5.58	10.7	13.3
BPS	1	-2.09	501	159	13.99	76	-6.41	8.5	13.8
BPS	10	-2.41	539	173	3.26	64	-7.20	8.8	12.0
BPS	10	-1.19	556	208	30.08	88	-7.20	9.1	13.0
BPS	10	-2.05	553	173	17.20	88	-7.20	9.0	13.4
BPS	10	-3.45	518	189	24.80	74	-6.37	9.3	13.1
BPS	10	-3.45	525	166	15.45	79	-7.20	8.8	13.9

Table A-21 (cont'd)

Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
BPS	50	-0.80	548	176	7.39	78	-6.35	8.8	13.1
BPS	50	-2.24	516	167	13.92	71	-5.60	10.1	11.6
BPS	50	-2.26	561	177	10.96	87	13.94	16.4	14.1
BPS	50	-1.25	506	169	3.92	61	-6.36	7.6	12.7
BES	1	-2.77	519	201	3.14	67	-6.45	8.8	14.3
BES	1	-2.19	484	195	8.34	73	-6.57	8.6	12.6
BES	10	-1.37	499	246	12.82	101	37.72	14.7	18.7
BES	10	1.21	477	225	6.32	94	32.54	11.3	16.5
BES	10	6.35	477	235	6.24	79	27.26	7.5	15.9
BES	10	8.75	476	247	15.91	88	33.20	9.3	17.3
BES	10	2.85	485	236	6.17	79	22.69	8.8	17.7
BES	50	4.10	478	245	16.74	93	37.78	10.5	18.6
BES	50	-1.15	504	244	6.24	86	17.20	9.9	18.4
BES	50	4.58	473	241	3.15	73	25.73	9.4	16.9
BES	50	3.22	492	256	12.84	84	36.54	11.9	15.5
BES	50	-0.50	473	235	4.30	82	23.79	6.3	17.7

Table A-22 Unprocessed data for Chapter 5: gases, fermentation, pH and ammonia

Additive	Conc. (mL/L)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	FOM[1], %	CH_4/FOM, µmoles /mg	pH	NH_4^+, ppm
Control	0	20.02	337	1878	59.2	2.463	7.01	182
Control	0	6.60	314	1577	63.0	2.167	6.98	192
Control	0	7.32	355	1724	60.9	2.534	6.95	-
Control	0	10.69	337	1928	60.0	2.428	6.94	195
C16:3	0.5	7.42	303	1576	59.7	2.201	6.94	181
C16:3	0.5	21.16	330	1720	60.9	2.350	6.84	195
C16:3	0.5	22.99	88	1310	42.1	0.909	7.08	148
C16:3	0.5	33.77	291	1828	59.3	2.139	6.93	191
C16:3	1	20.86	138	1232	49.6	1.204	6.86	179
C16:3	1	43.44	1	910	36.5	0.017	6.98	-
C16:3	1	14.57	220	1214	53.7	1.772	6.97	-
C16:3	1	7.74	272	1484	58.1	2.031	6.97	-
C16:3	2	36.25	1	727	35.7	0.016	7.02	155
C16:3	2	26.20	1	794	-	-	7.08	132
C16:3	2	49.65	37	1049	35.0	0.463	6.96	159
C16:3	2	118.49	1	1101	34.6	0.018	6.88	153
Olive oil	0.5	10.08	336	1920	56.9	2.561	7.01	192
Olive oil	0.5	10.92	335	1887	58.2	2.502	6.8	-
Olive oil	0.5	9.16	344	1995	61.0	2.448	7.07	200
Olive oil	0.5	-	-	-	58.3	0.000	6.94	196
Olive oil	1	24.12	337	1862	59.2	2.480	6.94	193
Olive oil	1	10.33	339	1653	59.6	2.458	6.92	-
Olive oil	1	0.00	347	1877	65.9	2.277	6.94	168
Olive oil	1	0.00	342	1848	64.7	2.290	6.75	-
Olive oil	2	11.56	344	1906	63.5	2.345	6.79	-
Olive oil	2	7.92	337	1935	-	-	7.06	186
Olive oil	2	7.75	341	2246	63.5	2.328	7.01	185
Olive oil	2	10.61	337	2200	61.0	2.399	6.96	204

[1]FOM = apparently fermented OM.

Table A-23 Unprocessed data for Chapter 5: VFA production

Additive	Conc. (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Control	0	0.00	1054	328	-0.54	110	0.00	22.9	20.3
Control	0	22.92	1135	347	-2.18	113	0.00	22.6	19.7
Control	0	0.00	1097	336	-2.04	111	0.00	20.0	20.0
Control	0	0.00	1088	331	-1.91	108	0.00	22.2	19.5
C16:3	0.5	9.42	1051	359	-3.79	112	0.00	18.4	18.8
C16:3	0.5	0.00	1086	348	-3.51	113	0.00	20.7	20.5
C16:3	0.5	10.23	657	377	-2.28	64	0.00	-2.2	22.8
C16:3	0.5	11.26	1018	343	-2.96	117	0.00	22.3	23.4
C16:3	1	0.00	749	400	-1.98	93	0.00	13.4	28.3
C16:3	1	9.96	513	398	-0.94	36	0.00	4.3	22.7
C16:3	1	0.00	904	343	-2.83	109	0.00	12.3	23.0
C16:3	1	0.00	1030	351	-3.34	111	0.00	11.3	18.6
C16:3	2	45.29	517	354	-2.52	51	23.96	6.8	19.2
C16:3	2	182.45	412	261	-4.20	43	15.54	4.1	16.9
C16:3	2	44.94	519	338	-3.68	48	16.77	4.1	20.1
C16:3	2	93.74	464	332	-4.82	58	21.50	5.1	35.6
Olive oil	0.5	0.00	1070	339	-2.55	94	0.00	12.1	1.1
Olive oil	0.5	0.00	1079	345	-5.04	97	0.00	16.2	6.0
Olive oil	0.5	0.00	1103	350	-2.89	106	0.00	20.5	17.3
Olive oil	0.5	37.01	1106	338	-4.69	88	0.00	10.1	16.1
Olive oil	1	54.90	1102	359	-4.14	96	0.00	13.6	2.3
Olive oil	1	28.68	1087	362	-5.04	100	0.00	15.1	16.1
Olive oil	1	22.02	1061	339	8.71	168	0.00	39.0	25.8
Olive oil	1	0.00	1023	328	5.31	181	31.77	47.5	12.7
Olive oil	2	44.63	1087	378	-9.66	155	0.00	28.0	0.6
Olive oil	2	59.55	1061	364	3.94	142	0.00	20.9	25.5
Olive oil	2	50.75	994	353	12.97	162	0.00	17.8	2.1

Table A-24 Unprocessed data for Chapter 6, Experiment 1: gases, pH and ammonia

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	pH	NH_4^+, mg/dL
Lumazine	0.0	crotonic acid	0	0.55	608	2707	7.00	25.3
Lumazine	0.0	crotonic acid	0	0.88	599	2401	6.91	25.5
Lumazine	0.0	crotonic acid	0	0.09	574	2811	6.99	25.7
Lumazine	0.0	crotonic acid	0	0.69	572	2564	7.08	25.2
Lumazine	0.0	crotonic acid	8	0.00	530	3340	7.03	25.1
Lumazine	0.0	crotonic acid	8	0.88	505	3094	7.08	25.1
Lumazine	0.0	crotonic acid	8	0.65	517	3122	7.14	25.6
Lumazine	0.0	crotonic acid	8	1.52	522	3193	7.00	27.1
Lumazine	0.3	crotonic acid	0	0.00	544	2736	7.16	25.3
Lumazine	0.3	crotonic acid	0	0.99	538	2701	7.12	26.9
Lumazine	0.3	crotonic acid	0	0.13	528	2775	6.94	27.5
Lumazine	0.3	crotonic acid	0	1.01	536	2771	7.00	28.6
Lumazine	0.3	crotonic acid	8	0.51	484	2977	6.99	27.0
Lumazine	0.3	crotonic acid	8	0.17	487	2978	7.00	27.2
Lumazine	0.3	crotonic acid	8	1.35	501	2981	7.09	26.5
Lumazine	0.3	crotonic acid	8	0.65	564	2857	6.96	28.2
Lumazine	0.6	crotonic acid	0	0.28	536	2009	6.95	25.7
Lumazine	0.6	crotonic acid	0	0.00	525	1995	6.94	27.3
Lumazine	0.6	crotonic acid	0	0.97	535	2384	7.03	26.4
Lumazine	0.6	crotonic acid	0	0.18	533	2168	6.95	26.7
Lumazine	0.6	crotonic acid	8	0.18	477	2568	6.93	25.0
Lumazine	0.6	crotonic acid	8	0.80	461	3288	7.00	24.8
Lumazine	0.6	crotonic acid	8	0.30	433	2982	6.99	25.0
Lumazine	0.6	crotonic acid	8	2.94	508	2984	6.86	26.4

Table A-25 Unprocessed data for Chapter 6, Experiment 1: VFA production

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Lumazine	0.0	crotonic acid	0	206.4	2205	663	12.2	157	0.0	44.8	64.9
Lumazine	0.0	crotonic acid	0	169.1	2119	648	12.2	146	0.0	44.0	64.5
Lumazine	0.0	crotonic acid	0	96.2	2107	646	11.7	143	0.0	46.6	63.8
Lumazine	0.0	crotonic acid	0	114.8	2215	686	12.6	146	0.0	43.8	72.5
Lumazine	0.0	crotonic acid	8	120.0	2294	648	11.2	295	0.0	468.2	66.4
Lumazine	0.0	crotonic acid	8	126.3	2238	638	12.6	277	0.0	457.0	58.5
Lumazine	0.0	crotonic acid	8	80.4	2252	638	13.4	291	0.0	466.0	59.5
Lumazine	0.0	crotonic acid	8	150.8	2318	653	13.7	312	0.0	475.2	69.0
Lumazine	0.3	crotonic acid	0	7.1	2132	636	36.3	138	0.0	30.7	65.4
Lumazine	0.3	crotonic acid	0	75.2	2134	650	51.6	144	0.0	38.0	66.1
Lumazine	0.3	crotonic acid	0	27.6	2156	615	34.7	140	0.0	41.9	71.6
Lumazine	0.3	crotonic acid	0	36.3	2153	632	52.4	162	4.4	41.9	66.5
Lumazine	0.3	crotonic acid	8	90.3	2250	626	47.7	300	0.0	456.7	60.3
Lumazine	0.3	crotonic acid	8	57.0	2244	601	46.7	290	0.0	456.0	66.1
Lumazine	0.3	crotonic acid	8	-18.9	2236	624	50.5	290	0.0	462.5	66.5
Lumazine	0.3	crotonic acid	8	33.0	2289	614	47.4	315	0.0	463.3	65.0
Lumazine	0.6	crotonic acid	0	-0.4	2099	620	65.9	144	0.0	36.1	67.9
Lumazine	0.6	crotonic acid	0	-8.8	2096	631	66.3	144	5.9	41.6	68.0
Lumazine	0.6	crotonic acid	0	24.8	2095	628	71.8	152	0.0	33.7	70.7
Lumazine	0.6	crotonic acid	0	9.7	2095	629	70.7	155	0.0	40.1	71.6
Lumazine	0.6	crotonic acid	8	-47.2	2084	554	52.4	268	0.0	452.9	63.8
Lumazine	0.6	crotonic acid	8	31.4	2069	560	60.6	267	0.0	447.0	67.3
Lumazine	0.6	crotonic acid	8	25.9	2123	556	60.9	274	0.0	447.1	57.0
Lumazine	0.6	crotonic acid	8	-0.5	2172	567	57.2	292	0.0	455.9	64.2

Table A-26 Unprocessed data for Chapter 6, Experiment 2: gases, pH and ammonia

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	pH	NH_4^+, mg/dL
Lumazine	0.0	3-butenoic acid	0	1.19	401	1951	6.87	30.0
Lumazine	0.0	3-butenoic acid	0	0.00	409	2150	6.77	31.6
Lumazine	0.0	3-butenoic acid	0	1.82	400	1954	6.8	28.3
Lumazine	0.0	3-butenoic acid	0	0.41	408	2143	6.79	31.4
Lumazine	0.0	3-butenoic acid	4	0.77	387	2050	6.87	27.8
Lumazine	0.0	3-butenoic acid	4	3.07	412	2280	6.88	31.2
Lumazine	0.0	3-butenoic acid	4	0.00	405	2293	6.78	30.8
Lumazine	0.0	3-butenoic acid	4	0.19	413	2422	6.83	30.5
Lumazine	0.3	3-butenoic acid	0	0.00	381	1892	6.83	30.0
Lumazine	0.3	3-butenoic acid	0	0.00	353	1322	6.85	31.0
Lumazine	0.3	3-butenoic acid	0	1.22	397	1987	6.77	30.8
Lumazine	0.3	3-butenoic acid	0	0.00	391	2101	6.8	30.9
Lumazine	0.3	3-butenoic acid	4	0.27	368	1998	6.72	26.9
Lumazine	0.3	3-butenoic acid	4	0.00	369	2165	6.75	28.0
Lumazine	0.3	3-butenoic acid	4	7.49	393	2472	6.73	28.6
Lumazine	0.3	3-butenoic acid	4	0.00	339	710	6.7	28.4
Lumazine	0.6	3-butenoic acid	0	1.55	353	2352	6.83	26.7
Lumazine	0.6	3-butenoic acid	0	1.47	343	2220	6.84	28.0
Lumazine	0.6	3-butenoic acid	0	1.78	333	1898	6.88	27.6
Lumazine	0.6	3-butenoic acid	0	2.44	339	1949	6.9	28.7
Lumazine	0.6	3-butenoic acid	4	0.00	302	1623	6.83	28.1
Lumazine	0.6	3-butenoic acid	4	1.30	307	1673	6.7	21.1
Lumazine	0.6	3-butenoic acid	4	0.00	313	1842	6.71	26.7
Lumazine	0.6	3-butenoic acid	4	3.53	312	1883	6.82	25.9

Table A-27 Unprocessed data for Chapter 6, Experiment 2: VFA production

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Lumazine	0.0	3-butenoic acid	0	0.0	1481	444	9.0	139	0.0	43.0	67.9
Lumazine	0.0	3-butenoic acid	0	0.0	1475	451	9.5	145	0.0	36.5	62.9
Lumazine	0.0	3-butenoic acid	0	0.0	1463	451	9.3	153	0.0	35.3	66.5
Lumazine	0.0	3-butenoic acid	0	0.0	1478	449	8.4	151	0.0	36.6	66.6
Lumazine	0.0	3-butenoic acid	4	30.4	1679	442	11.1	257	0.0	34.3	64.5
Lumazine	0.0	3-butenoic acid	4	32.4	1687	434	8.9	254	0.0	28.8	66.9
Lumazine	0.0	3-butenoic acid	4	29.5	1700	440	11.3	272	0.0	40.1	65.5
Lumazine	0.0	3-butenoic acid	4	32.4	1704	438	9.1	261	0.0	34.6	65.5
Lumazine	0.3	3-butenoic acid	0	0.0	1420	431	61.8	144	0.0	26.7	61.7
Lumazine	0.3	3-butenoic acid	0	2.0	1450	442	67.0	154	0.0	25.0	63.7
Lumazine	0.3	3-butenoic acid	0	0.0	1448	439	65.0	156	0.0	35.9	64.4
Lumazine	0.3	3-butenoic acid	0	1.5	1422	435	72.1	164	4.2	32.1	56.6
Lumazine	0.3	3-butenoic acid	4	6.1	1606	391	62.7	258	0.0	26.3	57.5
Lumazine	0.3	3-butenoic acid	4	29.0	1601	401	73.3	267	0.0	30.0	61.2
Lumazine	0.3	3-butenoic acid	4	40.6	1686	426	72.1	277	0.0	27.9	68.2
Lumazine	0.3	3-butenoic acid	4	20.3	1663	420	76.0	281	2.9	33.0	65.0
Lumazine	0.6	3-butenoic acid	0	0.0	1362	419	85.1	178	0.0	26.7	61.0
Lumazine	0.6	3-butenoic acid	0	0.0	1367	420	86.4	193	0.0	30.5	62.6
Lumazine	0.6	3-butenoic acid	0	0.0	1406	417	90.7	199	0.0	29.7	67.0
Lumazine	0.6	3-butenoic acid	0	0.0	1418	430	89.7	201	0.0	34.1	60.9
Lumazine	0.6	3-butenoic acid	4	33.2	1610	406	89.7	326	0.0	29.9	64.2
Lumazine	0.6	3-butenoic acid	4	32.0	1579	393	83.3	321	1.9	28.5	66.8
Lumazine	0.6	3-butenoic acid	4	31.7	1567	383	76.3	302	0.0	33.5	58.7
Lumazine	0.6	3-butenoic acid	4	40.3	1599	402	84.1	311	2.1	30.2	65.8

Table A-28 Unprocessed data for Chapter 6, Experiment 3: gases, pH and ammonia

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	pH	NH_4^+, mg/dL
Propynoic acid	0	crotonic acid	0	0.00	525	2740	6.95	24.5
Propynoic acid	0	crotonic acid	0	0.00	525	2564	6.84	26.1
Propynoic acid	0	crotonic acid	0	0.00	536	2443	6.84	26.6
Propynoic acid	0	crotonic acid	0	0.00	557	2753	6.84	27.0
Propynoic acid	0	crotonic acid	4	0.00	394	1430	6.91	25.8
Propynoic acid	0	crotonic acid	4	0.00	521	2738	6.73	26.1
Propynoic acid	0	crotonic acid	4	0.00	554	2749	6.81	26.6
Propynoic acid	0	crotonic acid	4	0.00	504	2875	6.87	26.0
Propynoic acid	2	crotonic acid	0	32.72	245	2236	6.74	21.8
Propynoic acid	2	crotonic acid	0	31.55	284	2257	6.81	22.5
Propynoic acid	2	crotonic acid	0	23.39	275	2536	6.66	22.1
Propynoic acid	2	crotonic acid	0	25.88	275	2462	6.87	23.5
Propynoic acid	2	crotonic acid	4	38.77	229	2211	6.63	22.3
Propynoic acid	2	crotonic acid	4	37.09	217	2397	6.69	22.7
Propynoic acid	2	crotonic acid	4	35.41	213	2151	6.6	22.7
Propynoic acid	2	crotonic acid	4	40.06	219	2530	6.61	23.3
Propynoic acid	4	crotonic acid	0	120.25	158	1908	6.77	21.4
Propynoic acid	4	crotonic acid	0	-	-	-	6.71	21.3
Propynoic acid	4	crotonic acid	0	138.42	176	2173	6.67	22.4
Propynoic acid	4	crotonic acid	0	138.90	177	1871	6.74	22.5
Propynoic acid	4	crotonic acid	4	130.28	152	2330	6.68	21.8
Propynoic acid	4	crotonic acid	4	104.70	152	1940	6.79	22.6
Propynoic acid	4	crotonic acid	4	108.62	156	2408	6.67	22.1
Propynoic acid	4	crotonic acid	4	100.24	160	2369	6.8	22.6

Table A-29 Unprocessed data for Chapter 6, Experiment 3: VFA production

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Propynoic acid	0	crotonic acid	0	42.6	1648	492	17.4	144	0.0	39.6	66.3
Propynoic acid	0	crotonic acid	0	17.6	1654	496	17.5	148	0.0	53.3	67.2
Propynoic acid	0	crotonic acid	0	13.1	1672	492	19.7	159	0.0	58.8	64.9
Propynoic acid	0	crotonic acid	0	6.4	1757	516	21.7	175	0.0	57.5	67.5
Propynoic acid	0	crotonic acid	4	16.5	1729	487	17.3	253	0.0	222.3	58.1
Propynoic acid	0	crotonic acid	4	-8.2	1717	485	15.0	256	0.0	219.8	59.4
Propynoic acid	0	crotonic acid	4	-8.2	1740	479	16.4	268	0.0	207.2	62.3
Propynoic acid	0	crotonic acid	4	-8.2	1754	479	16.2	276	0.0	215.6	63.6
Propynoic acid	2	crotonic acid	0	-8.2	1326	552	7.8	153	0.0	29.4	36.6
Propynoic acid	2	crotonic acid	0	-8.2	1312	536	8.2	150	0.0	17.3	31.7
Propynoic acid	2	crotonic acid	0	-8.2	1354	541	7.8	174	9.2	24.8	38.4
Propynoic acid	2	crotonic acid	0	-8.2	1341	531	9.3	190	14.7	23.4	40.1
Propynoic acid	2	crotonic acid	4	27.1	1338	545	9.7	255	18.3	221.3	33.9
Propynoic acid	2	crotonic acid	4	23.8	1381	547	8.4	277	0.0	215.3	31.8
Propynoic acid	2	crotonic acid	4	21.3	1363	560	4.4	243	0.0	225.8	36.8
Propynoic acid	2	crotonic acid	4	14.7	1342	534	6.9	255	8.0	224.4	35.9
Propynoic acid	4	crotonic acid	0	63.4	1219	552	0.6	124	10.6	13.0	22.7
Propynoic acid	4	crotonic acid	0	57.5	1188	552	0.3	121	13.9	10.4	21.7
Propynoic acid	4	crotonic acid	0	64.0	1221	560	3.4	134	16.8	12.5	25.1
Propynoic acid	4	crotonic acid	0	91.7	1181	538	7.1	141	17.7	12.5	-6.2
Propynoic acid	4	crotonic acid	4	62.7	1221	544	4.4	204	13.9	230.0	19.5
Propynoic acid	4	crotonic acid	4	40.8	1266	565	4.3	214	10.2	235.0	23.9
Propynoic acid	4	crotonic acid	4	45.4	1204	531	5.3	216	15.8	231.0	22.6
Propynoic acid	4	crotonic acid	4	69.1	1244	540	5.3	236	17.7	230.1	22.8

Table A-30 Unprocessed data for Chapter 6, Experiment 4: gases, pH and ammonia

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	pH	NH_4^+, mg/dL
Propynoic acid	0	3-butenoic acid	0	0.00	507	1811	6.63	42.4
Propynoic acid	0	3-butenoic acid	0	0.00	470	2161	6.75	44.1
Propynoic acid	0	3-butenoic acid	0	0.00	483	1962	6.76	43.3
Propynoic acid	0	3-butenoic acid	0	0.00	489	2000	6.69	41.9
Propynoic acid	0	3-butenoic acid	4	0.00	537	2610	6.81	38.5
Propynoic acid	0	3-butenoic acid	4	0.00	499	2256	6.66	42.2
Propynoic acid	0	3-butenoic acid	4	-	-	-	6.62	41.5
Propynoic acid	0	3-butenoic acid	4	0.00	488	1869	6.61	39.0
Propynoic acid	2	3-butenoic acid	0	45.68	368	2013	6.69	37.1
Propynoic acid	2	3-butenoic acid	0	44.62	338	1887	6.69	37.1
Propynoic acid	2	3-butenoic acid	0	46.82	295	1580	6.75	38.1
Propynoic acid	2	3-butenoic acid	0	55.71	262	1561	6.68	34.3
Propynoic acid	2	3-butenoic acid	4	-	-	-	6.66	34.5
Propynoic acid	2	3-butenoic acid	4	56.10	239	2032	6.61	34.9
Propynoic acid	2	3-butenoic acid	4	68.20	224	2119	6.63	33.8
Propynoic acid	2	3-butenoic acid	4	58.54	201	1585	6.53	33.4
Propynoic acid	4	3-butenoic acid	0	73.31	167	1577	6.65	33.2
Propynoic acid	4	3-butenoic acid	0	74.06	137	713	6.79	33.5
Propynoic acid	4	3-butenoic acid	0	75.13	161	1413	6.63	31.9
Propynoic acid	4	3-butenoic acid	0	73.53	153	1855	6.65	32.8
Propynoic acid	4	3-butenoic acid	4	75.21	165	2305	6.65	31.5
Propynoic acid	4	3-butenoic acid	4	70.73	148	1673	6.57	27.7
Propynoic acid	4	3-butenoic acid	4	92.19	159	2063	6.56	32.7
Propynoic acid	4	3-butenoic acid	4	80.91	151	1945	6.59	31.4

Table A-31 Unprocessed data for Chapter 6, Experiment 4: VFA production

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Propynoic acid	0	3-butenoic acid	0	0.0	1616	499	1.1	117	0.0	38.4	58.8
Propynoic acid	0	3-butenoic acid	0	0.0	1682	523	0.4	127	0.0	42.0	64.9
Propynoic acid	0	3-butenoic acid	0	0.0	1721	538	2.9	127	0.0	42.2	64.9
Propynoic acid	0	3-butenoic acid	0	0.0	1729	534	21.8	136	0.0	38.4	59.8
Propynoic acid	0	3-butenoic acid	4	151.7	1441	552	-6.1	286	0.0	14.0	22.8
Propynoic acid	0	3-butenoic acid	4	0.0	1943	501	0.4	247	0.0	39.6	67.0
Propynoic acid	0	3-butenoic acid	4	69.2	1928	502	-6.7	248	0.0	40.7	63.0
Propynoic acid	0	3-butenoic acid	4	75.2	1970	511	-5.7	249	0.0	38.1	66.5
Propynoic acid	2	3-butenoic acid	0	0.0	1411	497	17.0	196	0.0	21.5	45.3
Propynoic acid	2	3-butenoic acid	0	0.0	1418	510	-0.8	151	0.0	22.3	39.6
Propynoic acid	2	3-butenoic acid	0	0.0	1432	499	-2.4	142	0.0	23.4	36.8
Propynoic acid	2	3-butenoic acid	0	0.0	1432	561	-0.9	149	0.0	17.0	30.8
Propynoic acid	2	3-butenoic acid	4	117.7	1500	536	-0.4	284	0.0	26.0	27.4
Propynoic acid	2	3-butenoic acid	4	106.5	1521	550	28.2	324	0.0	28.5	39.9
Propynoic acid	2	3-butenoic acid	4	111.6	1548	536	-8.0	279	0.0	19.4	38.1
Propynoic acid	2	3-butenoic acid	4	116.0	1569	578	-0.6	292	0.0	26.0	39.7
Propynoic acid	4	3-butenoic acid	0	92.5	1254	561	-3.6	156	0.0	13.0	-1.2
Propynoic acid	4	3-butenoic acid	0	85.3	1270	573	-5.7	143	0.0	0.1	-1.6
Propynoic acid	4	3-butenoic acid	0	80.5	1271	593	41.4	236	0.0	32.9	8.5
Propynoic acid	4	3-butenoic acid	0	90.3	1276	579	-5.7	155	0.0	15.5	1.8
Propynoic acid	4	3-butenoic acid	4	183.8	1399	534	12.6	334	0.0	7.1	21.0
Propynoic acid	4	3-butenoic acid	4	112.6	1389	536	-8.6	280	0.0	10.6	18.0
Propynoic acid	4	3-butenoic acid	4	155.9	1432	587	29.9	315	0.0	9.9	9.2
Propynoic acid	4	3-butenoic acid	4	191.9	1447	549	13.4	288	0.0	-0.3	20.0

Table A-32 Unprocessed data for Chapter 6, Experiment 5: gases, pH and ammonia

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	pH	NH_4^+, mg/dL
Ethyl 2-butynoate	0	crotonic acid	0	6.09	645	3090	6.80	37.6
Ethyl 2-butynoate	0	crotonic acid	0	7.83	621	2904	6.77	34.8
Ethyl 2-butynoate	0	crotonic acid	0	11.15	703	3651	6.76	36.3
Ethyl 2-butynoate	0	crotonic acid	0	3.33	656	3499	6.71	30.9
Ethyl 2-butynoate	0	crotonic acid	4	4.80	620	3356	6.67	31.6
Ethyl 2-butynoate	0	crotonic acid	4	6.53	680	3841	6.75	32.3
Ethyl 2-butynoate	0	crotonic acid	4	8.35	668	3520	6.71	28.7
Ethyl 2-butynoate	0	crotonic acid	4	7.16	603	3394	6.75	34.3
Ethyl 2-butynoate	4	crotonic acid	0	31.51	427	2973	6.79	24.1
Ethyl 2-butynoate	4	crotonic acid	0	36.21	346	2974	6.66	28.8
Ethyl 2-butynoate	4	crotonic acid	0	41.28	347	3085	6.71	27.8
Ethyl 2-butynoate	4	crotonic acid	0	38.77	305	2803	6.71	27.6
Ethyl 2-butynoate	4	crotonic acid	4	28.89	378	3161	6.69	25.7
Ethyl 2-butynoate	4	crotonic acid	4	34.22	286	1808	6.61	26.8
Ethyl 2-butynoate	4	crotonic acid	4	23.75	344	2897	6.80	25.9
Ethyl 2-butynoate	4	crotonic acid	4	25.62	422	2988	6.65	29.0
Ethyl 2-butynoate	8	crotonic acid	0	-	-	-	6.81	25.1
Ethyl 2-butynoate	8	crotonic acid	0	42.96	68	2324	6.80	25.4
Ethyl 2-butynoate	8	crotonic acid	0	51.09	53	1606	6.85	24.7
Ethyl 2-butynoate	8	crotonic acid	0	47.44	53	1794	6.79	29.2
Ethyl 2-butynoate	8	crotonic acid	4	-	-	-	6.74	23.1
Ethyl 2-butynoate	8	crotonic acid	4	44.74	37	2105	6.87	26.3
Ethyl 2-butynoate	8	crotonic acid	4	48.32	56	2460	6.80	26.4
Ethyl 2-butynoate	8	crotonic acid	4	38.12	58	2221	6.76	25.1

Table A-33 Unprocessed data for Chapter 6, Experiment 5: VFA production

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Ethyl 2-butynoate	0	crotonic acid	0	0.0	2361	682	36.4	239	0.0	55.2	79.4
Ethyl 2-butynoate	0	crotonic acid	0	3.7	2365	686	32.2	228	0.0	55.6	74.7
Ethyl 2-butynoate	0	crotonic acid	0	2.8	2412	725	37.8	245	0.0	57.8	76.4
Ethyl 2-butynoate	0	crotonic acid	0	1.9	2443	704	32.7	245	0.0	74.7	92.7
Ethyl 2-butynoate	0	crotonic acid	4	8.9	2349	660	27.8	311	0.0	139.9	74.2
Ethyl 2-butynoate	0	crotonic acid	4	0.0	2420	675	29.7	319	0.0	135.8	78.2
Ethyl 2-butynoate	0	crotonic acid	4	9.6	2419	677	34.0	338	0.0	145.0	72.1
Ethyl 2-butynoate	0	crotonic acid	4	4.7	2499	706	32.8	341	0.0	141.1	81.7
Ethyl 2-butynoate	4	crotonic acid	0	152.0	2085	748	91.9	224	231.5	27.6	72.6
Ethyl 2-butynoate	4	crotonic acid	0	162.8	1959	732	90.5	233	311.8	42.2	97.2
Ethyl 2-butynoate	4	crotonic acid	0	155.9	1954	744	82.8	218	249.6	29.0	73.1
Ethyl 2-butynoate	4	crotonic acid	0	223.5	2057	901	144.5	296	389.8	35.9	79.9
Ethyl 2-butynoate	4	crotonic acid	4	181.2	2020	680	42.0	350	234.9	120.1	69.5
Ethyl 2-butynoate	4	crotonic acid	4	218.7	2002	670	46.0	351	235.6	107.9	73.6
Ethyl 2-butynoate	4	crotonic acid	4	184.6	2045	706	77.9	333	195.8	99.3	75.9
Ethyl 2-butynoate	4	crotonic acid	4	211.0	2187	728	88.1	346	173.3	96.2	75.6
Ethyl 2-butynoate	8	crotonic acid	0	496.8	1371	539	202.4	292	503.2	46.4	33.4
Ethyl 2-butynoate	8	crotonic acid	0	450.3	1484	634	221.3	286	470.1	29.2	38.2
Ethyl 2-butynoate	8	crotonic acid	0	519.9	1390	535	197.2	271	422.3	35.4	36.6
Ethyl 2-butynoate	8	crotonic acid	0	473.2	1420	573	223.9	294	485.1	40.9	38.1
Ethyl 2-butynoate	8	crotonic acid	4	632.8	1301	482	173.6	315	392.2	147.0	31.6
Ethyl 2-butynoate	8	crotonic acid	4	572.1	1470	572	215.3	352	457.0	147.7	31.6
Ethyl 2-butynoate	8	crotonic acid	4	572.2	1538	617	191.8	350	454.3	129.7	39.7
Ethyl 2-butynoate	8	crotonic acid	4	593.1	1484	551	209.9	347	440.4	148.1	36.2

Table A-34 Unprocessed data for Chapter 6, Experiment 6: gases, pH and ammonia

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	H_2, µmoles	CH_4, µmoles	CO_2, µmoles	pH	NH_4^+, mg/dL
Ethyl 2-butynoate	0	3-Butenoic acid	0	0.00	514	2112	6.80	27.1
Ethyl 2-butynoate	0	3-butenoic acid	0	0.00	510	2031	6.88	19.5
Ethyl 2-butynoate	0	3-butenoic acid	0	0.00	503	1726	6.84	28.6
Ethyl 2-butynoate	0	3-butenoic acid	0	0.00	445	861	6.86	21.1
Ethyl 2-butynoate	0	3-butenoic acid	4	0.00	520	2100	6.81	27.6
Ethyl 2-butynoate	0	3-butenoic acid	4	0.00	537	2472	6.76	27.6
Ethyl 2-butynoate	0	3-butenoic acid	4	0.00	496	1800	6.75	19.9
Ethyl 2-butynoate	0	3-butenoic acid	4	0.00	548	2586	6.92	19.6
Ethyl 2-butynoate	4	3-butenoic acid	0	8.72	269	1805	6.71	21.3
Ethyl 2-butynoate	4	3-butenoic acid	0	0.76	299	2078	6.85	15.6
Ethyl 2-butynoate	4	3-butenoic acid	0	4.99	246	1694	6.85	21.8
Ethyl 2-butynoate	4	3-butenoic acid	0	14.49	269	2004	6.91	22.8
Ethyl 2-butynoate	4	3-butenoic acid	4	5.05	265	2237	6.73	20.5
Ethyl 2-butynoate	4	3-butenoic acid	4	13.67	247	2146	6.78	21.0
Ethyl 2-butynoate	4	3-butenoic acid	4	12.64	260	1916	6.75	13.6
Ethyl 2-butynoate	4	3-butenoic acid	4	25.30	235	2447	6.85	13.6
Ethyl 2-butynoate	8	3-butenoic acid	0	30.88	0	1093	6.87	20.8
Ethyl 2-butynoate	8	3-butenoic acid	0	35.91	0	930	6.92	20.7
Ethyl 2-butynoate	8	3-butenoic acid	0	39.42	0	1176	6.80	20.8
Ethyl 2-butynoate	8	3-butenoic acid	0	38.65	0	1811	6.87	20.7
Ethyl 2-butynoate	8	3-butenoic acid	4	38.48	0	1340	6.85	20.2
Ethyl 2-butynoate	8	3-butenoic acid	4	40.06	0	1713	6.76	20.1
Ethyl 2-butynoate	8	3-butenoic acid	4	44.96	0	1498	6.90	19.6
Ethyl 2-butynoate	8	3-butenoic acid	4	45.12	0	1879	6.76	19.7

Table A-35 Unprocessed data for Chapter 6, Experiment 6: VFA production

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	Formate, µmoles	Acetate, µmoles	Propionate, µmoles	Isobutyrate, µmoles	Butyrate, µmoles	Ethanol, µmoles	Isovalerate, µmoles	Valerate, µmoles
Ethyl 2-butynoate	0	3-Butenoic acid	0	-1.9	1764	607	25.2	133	-2.4	30.5	61.1
Ethyl 2-butynoate	0	3-butenoic acid	0	-1.9	1787	613	25.1	140	-2.4	27.2	64.9
Ethyl 2-butynoate	0	3-butenoic acid	0	13.0	1872	655	26.6	155	1.2	36.0	67.6
Ethyl 2-butynoate	0	3-butenoic acid	0	-1.9	1783	610	33.4	154	0.7	31.6	65.2
Ethyl 2-butynoate	0	3-butenoic acid	4	16.6	1979	590	25.6	254	-2.4	32.3	65.3
Ethyl 2-butynoate	0	3-butenoic acid	4	26.0	1891	562	22.9	237	-2.4	31.2	62.0
Ethyl 2-butynoate	0	3-butenoic acid	4	7.1	2020	602	23.9	258	1.3	37.2	61.1
Ethyl 2-butynoate	0	3-butenoic acid	4	34.5	2032	580	23.6	273	1.1	36.5	65.6
Ethyl 2-butynoate	4	3-butenoic acid	0	475.3	1512	627	43.3	139	98.1	-1.3	55.8
Ethyl 2-butynoate	4	3-butenoic acid	0	449.8	1536	622	40.0	142	111.2	11.6	59.8
Ethyl 2-butynoate	4	3-butenoic acid	0	503.3	1453	607	45.3	136	157.9	5.8	53.7
Ethyl 2-butynoate	4	3-butenoic acid	0	478.3	1511	628	52.8	152	161.4	9.3	62.7
Ethyl 2-butynoate	4	3-butenoic acid	4	459.0	1652	617	36.5	266	98.3	1.8	61.4
Ethyl 2-butynoate	4	3-butenoic acid	4	480.8	1614	610	47.4	266	105.7	-1.2	56.8
Ethyl 2-butynoate	4	3-butenoic acid	4	474.3	1669	622	50.9	263	133.7	-1.3	56.7
Ethyl 2-butynoate	4	3-butenoic acid	4	562.2	1632	619	50.2	289	133.3	4.6	66.1
Ethyl 2-butynoate	8	3-butenoic acid	0	881.3	1184	553	149.8	182	294.8	-1.3	24.7
Ethyl 2-butynoate	8	3-butenoic acid	0	869.0	1109	500	146.5	184	251.8	3.0	22.0
Ethyl 2-butynoate	8	3-butenoic acid	0	921.6	1137	516	154.8	188	304.6	2.3	21.6
Ethyl 2-butynoate	8	3-butenoic acid	0	913.6	1151	483	166.0	195	311.5	9.5	25.8
Ethyl 2-butynoate	8	3-butenoic acid	4	894.3	1221	447	155.5	303	263.5	-0.3	-0.8
Ethyl 2-butynoate	8	3-butenoic acid	4	909.0	1296	534	146.6	296	269.0	5.1	29.7
Ethyl 2-butynoate	8	3-butenoic acid	4	896.8	1267	506	149.3	287	276.4	10.0	25.5
Ethyl 2-butynoate	8	3-butenoic acid	4	927.0	1270	502	158.6	302	283.9	10.1	22.0

Table A-36 Unprocessed data for Chapter 7, Experiment 1

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMNS[7], g/kg	pH
Lumazine	0.0	crotonic acid	0	44.1	53.0	43.9	0.54	0.048	0.225	20.0	6.74
Lumazine	0.0	crotonic acid	0	40.0	54.6	39.3	0.51	0.047	0.231	21.5	6.73
Lumazine	0.0	crotonic acid	0	40.3	54.9	38.0	0.52	0.048	0.235	21.6	6.68
Lumazine	0.0	crotonic acid	8	42.7	50.3	44.0	0.57	0.049	0.246	21.2	6.64
Lumazine	0.0	crotonic acid	8	43.8	50.7	42.6	0.57	0.050	0.240	21.0	6.64
Lumazine	0.0	crotonic acid	8	38.7	48.8	38.5	0.54	0.051	0.257	24.2	6.58
Lumazine	0.3	crotonic acid	0	40.2	50.9	42.4	0.53	0.049	0.242	22.3	6.72
Lumazine	0.3	crotonic acid	0	39.8	50.6	43.4	0.58	0.050	0.265	23.1	6.68
Lumazine	0.3	crotonic acid	0	34.0	44.2	40.5	0.60	0.054	0.322	28.9	6.68
Lumazine	0.3	crotonic acid	8	40.5	47.5	38.1	0.55	0.054	0.251	24.2	6.66
Lumazine	0.3	crotonic acid	8	45.1	48.4	43.2	0.57	0.055	0.231	22.2	6.57
Lumazine	0.3	crotonic acid	8	45.6	49.6	45.3	0.51	0.053	0.206	21.3	6.6
Lumazine	0.6	crotonic acid	0	40.3	50.0	40.1	0.58	0.057	0.262	25.8	6.76
Lumazine	0.6	crotonic acid	0	37.2	46.6	36.5	0.60	0.057	0.297	28.0	6.69
Lumazine	0.6	crotonic acid	0	41.4	51.9	38.8	0.56	0.052	0.247	23.1	6.66
Lumazine	0.6	crotonic acid	8	37.7	38.8	36.8	0.61	0.060	0.296	29.0	6.65
Lumazine	0.6	crotonic acid	8	40.7	44.5	39.4	0.59	0.058	0.264	26.0	6.58
Lumazine	0.6	crotonic acid	8	41.7	49.0	37.7	0.58	0.056	0.254	24.6	-

[1]OMD = true OM degradation; [2]ND = true N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMNS = efficiency of microbial N production (substrate OM basis).

Table A-37 Unprocessed data for Chapter 7, Experiment 2

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMNS[7], g/kg	pH
Lumazine	0.0	3-butenoic acid	0	41.5	67.9	40.5	0.53	0.051	0.233	22.4	6.75
Lumazine	0.0	3-butenoic acid	0	44.7	67.9	41.0	0.45	0.046	0.186	18.6	6.73
Lumazine	0.0	3-butenoic acid	0	47.3	72.7	41.5	0.44	0.040	0.170	15.6	6.72
Lumazine	0.0	3-butenoic acid	4	44.1	74.7	39.5	0.52	0.047	0.217	19.4	6.70
Lumazine	0.0	3-butenoic acid	4	46.6	71.1	42.6	0.56	0.052	0.221	20.4	6.69
Lumazine	0.0	3-butenoic acid	4	45.8	75.1	40.5	0.49	0.045	0.196	18.1	6.66
Lumazine	0.3	3-butenoic acid	0	45.5	69.8	41.6	0.54	0.054	0.219	21.9	6.70
Lumazine	0.3	3-butenoic acid	0	43.6	62.7	40.8	0.58	0.056	0.245	23.6	6.74
Lumazine	0.3	3-butenoic acid	0	47.1	69.7	43.0	0.56	0.055	0.218	21.5	6.75
Lumazine	0.3	3-butenoic acid	4	46.5	76.2	40.3	0.55	0.054	0.218	21.3	6.68
Lumazine	0.3	3-butenoic acid	4	45.0	71.7	38.9	0.59	0.057	0.240	23.1	6.69
Lumazine	0.3	3-butenoic acid	4	47.0	72.8	40.9	0.57	0.053	0.220	20.6	6.69
Lumazine	0.6	3-butenoic acid	0	43.6	61.7	40.5	0.54	0.054	0.226	22.6	6.74
Lumazine	0.6	3-butenoic acid	0	42.2	62.1	39.0	0.55	0.054	0.240	23.6	6.75
Lumazine	0.6	3-butenoic acid	0	42.6	57.9	38.9	0.56	0.055	0.240	23.5	6.74
Lumazine	0.6	3-butenoic acid	4	45.1	65.7	40.5	0.58	0.055	0.236	22.5	6.72
Lumazine	0.6	3-butenoic acid	4	43.8	60.7	36.7	0.60	0.056	0.250	23.3	6.70
Lumazine	0.6	3-butenoic acid	4	43.9	59.7	41.4	0.67	0.059	0.278	24.7	6.72

[1]OMD = true OM degradation; [2]ND = true N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMNS = efficiency of microbial N production (substrate OM basis).

Table A-38 Unprocessed data for Chapter 7, Experiment 3

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[9], g/kg	pH
Propynoic acid	0.0	crotonic acid	0	38.2	48.0	37.8	0.71	0.067	0.342	0.342	32.3	32.3	6.71
Propynoic acid	0.0	crotonic acid	0	39.2	41.0	36.2	0.63	0.065	0.296	0.296	30.4	30.4	6.70
Propynoic acid	0.0	crotonic acid	0	40.6	42.2	36.8	0.74	0.059	0.332	0.332	26.5	26.5	6.69
Propynoic acid	0.0	crotonic acid	4	37.8	28.5	32.5	0.93	0.074	0.450	0.398	36.0	31.9	6.64
Propynoic acid	0.0	crotonic acid	4	34.6	26.0	29.9	0.76	0.069	0.405	0.354	36.5	32.0	6.66
Propynoic acid	0.0	crotonic acid	4	35.0	33.0	30.4	0.72	0.069	0.377	0.330	35.9	31.5	6.64
Propynoic acid	0.3	crotonic acid	0	25.9	21.6	23.3	0.77	0.085	0.543	0.543	59.8	59.7	6.72
Propynoic acid	0.3	crotonic acid	0	27.1	16.6	27.0	0.84	0.083	0.567	0.566	55.9	55.9	6.70
Propynoic acid	0.3	crotonic acid	0	27.8	23.8	26.4	0.86	0.082	0.568	0.568	54.1	54.0	6.75
Propynoic acid	0.3	crotonic acid	4	28.5	20.7	23.4	0.93	0.088	0.597	0.538	56.7	51.1	6.73
Propynoic acid	0.3	crotonic acid	4	26.6	25.5	20.5	0.87	0.087	0.599	0.538	59.8	53.7	6.75
Propynoic acid	0.3	crotonic acid	4	31.0	23.7	29.0	0.94	0.084	0.554	0.496	49.9	44.7	6.66
Propynoic acid	0.6	crotonic acid	0	29.4	1.4	29.8	0.89	0.086	0.554	0.552	53.5	53.3	6.74
Propynoic acid	0.6	crotonic acid	0	25.5	1.2	22.0	0.86	0.080	0.617	0.617	57.5	57.5	6.67

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); [8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).

Table A-38 (cont'd)

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[9], g/kg	pH
Propynoic acid	0.6	crotonic acid	0	23.0	-	23.1	0.87	0.080	0.691	0.690	64.0	63.8	6.71
Propynoic acid	0.6	crotonic acid	4	30.0	0.8	26.0	0.93	0.084	0.568	0.510	51.0	45.8	6.63
Propynoic acid	0.6	crotonic acid	4	25.0	2.3	22.5	0.77	0.081	0.567	0.500	59.7	52.6	6.64
Propynoic acid	0.6	crotonic acid	4	17.7	-	27.0	0.90	0.086	0.930	0.778	88.6	74.1	6.60

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); [8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).

Table A-39 Unprocessed data for Chapter 7, Experiment 4

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[9], g/kg	pH
Propynoic acid	0.0	3-butenoic acid	0	33.9	52.7	28.2	0.57	0.051	0.306	0.306	27.7	27.7	6.89
Propynoic acid	0.0	3-butenoic acid	0	29.9	41.5	24.4	0.60	0.053	0.367	0.367	32.7	32.7	6.84
Propynoic acid	0.0	3-butenoic acid	0	32.0	39.7	26.4	0.62	0.055	0.354	0.354	31.5	31.5	6.79
Propynoic acid	0.0	3-butenoic acid	4	24.3	38.3	22.0	0.65	0.059	0.491	0.408	44.2	36.8	6.82
Propynoic acid	0.0	3-butenoic acid	4	33.6	44.7	26.9	0.62	0.057	0.339	0.296	31.3	27.3	6.69
Propynoic acid	0.0	3-butenoic acid	4	34.4	39.3	30.9	0.61	0.058	0.324	0.283	31.1	27.2	6.79
Propynoic acid	0.3	3-butenoic acid	0	24.0	39.1	16.7	0.71	0.072	0.544	0.536	55.0	54.2	6.89
Propynoic acid	0.3	3-butenoic acid	0	25.3	39.7	15.2	0.80	0.073	0.580	0.572	53.0	52.4	6.77
Propynoic acid	0.3	3-butenoic acid	0	22.6	40.8	14.1	0.75	0.073	0.604	0.596	59.4	58.5	6.79
Propynoic acid	0.3	3-butenoic acid	4	30.6	52.7	22.1	0.78	0.074	0.466	0.397	44.2	37.7	6.81
Propynoic acid	0.3	3-butenoic acid	4	27.9	48.4	19.1	0.87	0.081	0.572	0.480	52.9	44.4	6.71
Propynoic acid	0.3	3-butenoic acid	4	31.3	45.7	22.2	0.82	0.079	0.477	0.408	46.1	39.4	6.72
Propynoic acid	0.6	3-butenoic acid	0	28.7	42.6	16.2	0.72	0.069	0.460	0.448	44.1	42.9	6.88
Propynoic acid	0.6	3-butenoic acid	0	24.7	36.7	20.0	0.70	0.068	0.519	0.503	50.7	49.2	6.73

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); **[8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).**

Table A-39 (cont'd)

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[9], g/kg	pH
Propynoic acid	0.6	3-butenoic acid	0	23.7	18.2	15.3	0.75	0.073	0.584	0.565	56.5	54.7	6.75
Propynoic acid	0.6	3-butenoic acid	4	27.9	38.9	18.1	0.82	0.073	0.536	0.445	47.6	39.5	6.78
Propynoic acid	0.6	3-butenoic acid	4	28.3	31.0	22.5	0.83	0.077	0.538	0.449	49.7	41.5	6.73
Propynoic acid	0.6	3-butenoic acid	4	25.2	27.7	16.5	0.86	0.081	0.626	0.511	58.5	47.8	6.72

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); [8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).

Table A-40 Unprocessed data for Chapter 7, Experiment 5

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[9], g/kg	pH
Ethyl 2-butynoate	0.0	crotonic acid	0	33.9	52.7	28.2	0.57	0.051	0.306	0.306	27.7	27.7	6.89
Ethyl 2-butynoate	0.0	crotonic acid	0	29.9	41.5	24.4	0.60	0.053	0.367	0.367	32.7	32.7	6.84
Ethyl 2-butynoate	0.0	crotonic acid	0	32.0	39.7	26.4	0.62	0.055	0.354	0.354	31.5	31.5	6.79
Ethyl 2-butynoate	0.0	crotonic acid	8	24.3	38.3	22.0	0.65	0.059	0.491	0.408	44.2	36.8	6.82
Ethyl 2-butynoate	0.0	crotonic acid	8	33.6	44.7	26.9	0.62	0.057	0.339	0.296	31.3	27.3	6.69
Ethyl 2-butynoate	0.0	crotonic acid	8	34.4	39.3	30.9	0.61	0.058	0.324	0.283	31.1	27.2	6.79
Ethyl 2-butynoate	0.3	crotonic acid	0	24.0	39.1	16.7	0.71	0.072	0.544	0.536	55.0	54.2	6.89
Ethyl 2-butynoate	0.3	crotonic acid	0	25.3	39.7	15.2	0.80	0.073	0.580	0.572	53.0	52.4	6.77
Ethyl 2-butynoate	0.3	crotonic acid	0	22.6	40.8	14.1	0.75	0.073	0.604	0.596	59.4	58.5	6.79
Ethyl 2-butynoate	0.3	crotonic acid	8	30.6	52.7	22.1	0.78	0.074	0.466	0.397	44.2	37.7	6.81
Ethyl 2-butynoate	0.3	crotonic acid	8	27.9	48.4	19.1	0.87	0.081	0.572	0.480	52.9	44.4	6.71
Ethyl 2-butynoate	0.3	crotonic acid	8	31.3	45.7	22.2	0.82	0.079	0.477	0.408	46.1	39.4	6.72
Ethyl 2-butynoate	0.6	crotonic acid	0	28.7	42.6	16.2	0.72	0.069	0.460	0.448	44.1	42.9	6.88
Ethyl 2-butynoate	0.6	crotonic acid	0	24.7	36.7	20.0	0.70	0.068	0.519	0.503	50.7	49.2	6.73
Ethyl 2-butynoate	0.6	crotonic acid	0	23.7	18.2	15.3	0.75	0.073	0.584	0.565	56.5	54.7	6.75

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); [8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).

Table A-40 (cont'd)

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[9], g/kg	pH
Ethyl 2-butynoate	0.6	crotonic acid	8	27.9	38.9	18.1	0.82	0.073	0.536	0.445	47.6	39.5	6.78
Ethyl 2-butynoate	0.6	crotonic acid	8	28.3	31.0	22.5	0.83	0.077	0.538	0.449	49.7	41.5	6.73
Ethyl 2-butynoate	0.6	crotonic acid	8	25.2	27.7	16.5	0.86	0.081	0.626	0.511	58.5	47.8	6.72

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); [8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).

Table A-41 Unprocessed data for Chapter 7, Experiment 6

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[9], g/kg	pH
Ethyl 2-butynoate	0.0	3-butenoic acid	0	45.1	74.3	40.8	0.53	0.048	0.215	0.215	19.4	19.4	6.78
Ethyl 2-butynoate	0.0	3-butenoic acid	0	42.9	69.3	39.1	0.50	0.045	0.214	0.214	19.4	19.4	6.74
Ethyl 2-butynoate	0.0	3-butenoic acid	0	43.7	73.2	38.6	0.42	0.042	0.177	0.177	17.7	17.7	6.75
Ethyl 2-butynoate	0.0	3-butenoic acid	4	45.1	77.7	39.7	0.54	0.048	0.219	0.198	19.5	17.7	6.73
Ethyl 2-butynoate	0.0	3-butenoic acid	4	20.1	59.9	12.6	0.70	0.063	0.635	0.513	57.1	46.1	6.70
Ethyl 2-butynoate	0.0	3-butenoic acid	4	42.4	71.7	39.1	0.47	0.042	0.201	0.181	18.0	16.2	6.71
Ethyl 2-butynoate	0.3	3-butenoic acid	0	45.0	69.4	38.5	0.75	0.072	0.306	0.269	29.3	25.8	6.74
Ethyl 2-butynoate	0.3	3-butenoic acid	0	44.5	64.2	39.8	0.71	0.073	0.293	0.257	30.1	26.5	6.73
Ethyl 2-butynoate	0.3	3-butenoic acid	0	44.8	68.7	40.2	0.67	0.069	0.275	0.241	28.1	24.7	6.78
Ethyl 2-butynoate	0.3	3-butenoic acid	4	49.4	74.3	40.6	0.79	0.074	0.294	0.241	27.5	22.5	6.77
Ethyl 2-butynoate	0.3	3-butenoic acid	4	42.7	66.6	38.3	0.71	0.070	0.304	0.242	30.2	24.0	6.65
Ethyl 2-butynoate	0.3	3-butenoic acid	4	44.9	67.3	41.1	0.69	0.068	0.280	0.225	27.8	22.3	6.82
Ethyl 2-butynoate	0.6	3-butenoic acid	0	39.1	55.6	34.2	0.78	0.077	0.365	0.277	36.2	27.5	6.70
Ethyl 2-butynoate	0.6	3-butenoic acid	0	39.1	61.0	31.0	0.67	0.063	0.311	0.236	29.6	22.4	6.68
Ethyl 2-butynoate	0.6	3-butenoic acid	0	35.8	56.6	30.0	0.72	0.068	0.371	0.275	34.6	25.7	6.70
Ethyl 2-butynoate	0.6	3-butenoic acid	4	40.0	54.7	36.1	0.67	0.069	0.308	0.216	31.6	22.1	6.64

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); [8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).

Table A-41 (cont'd)

Inhibitor	Conc. (mM)	Butyrate enhancer	Conc (mM)	OMD[1], %	ND[2], %	NDFD[3], %	MOM[4], g	MN[5], g	EMOMS[6], g/kg	EMOMT[7], g/kg	EMNS[8], g/kg	EMNT[8], g/kg	pH
Ethyl 2-butynoate	0.6	3-butenoic acid	4	39.6	52.3	32.9	0.66	0.067	0.307	0.214	31.0	21.6	6.64
Ethyl 2-butynoate	0.6	3-butenoic acid	4	39.8	59.5	35.7	0.66	0.067	0.305	0.213	30.6	21.4	6.62

[1]OMD = OM degradation; [2]ND = N degradation; [3]NDFD = NDF degradation; [4]MOM = microbial OM production; [5]MN = microbial N production; [6]EMOMS = efficiency of microbial OM production (substrate OM basis); [7]EMOMT = efficiency of microbial OM production (total OM basis); [8]EMNS = efficiency of microbial N production (substrate OM basis); [9]EMNT = Efficiency of microbial N production (total OM basis).

Table A-42 Unprocessed data for Chapter 8

Tube	Species	Days	Treatment	Conc	Area	CH_4, µmoles
1	*M. ruminantium*	4	control	0	47.7471	11.95
2	*M. ruminantium*	4	control	0	48.1681	12.05
3	*M. ruminantium*	4	control	0	45.9153	11.49
10	*M. ruminantium*	4	BES	10	21.3354	5.34
11	*M. ruminantium*	4	BES	10	34.3805	8.60
12	*M. ruminantium*	4	BES	10	38.0693	9.52
19	*M. ruminantium*	4	BES	50	0.7147	0.18
20	*M. ruminantium*	4	BES	50	0.9364	0.23
21	*M. ruminantium*	4	BES	50	11.3404	2.84
28	*M. ruminantium*	4	BES	250	0	0.00
29	*M. ruminantium*	4	BES	250	0	0.00
30	*M. ruminantium*	4	BES	250	0	0.00
37	*M. ruminantium*	4	BPS	10	51.7164	12.90
38	*M. ruminantium*	4	BPS	10	53.2547	13.28
39	*M. ruminantium*	4	BPS	10	53.7936	13.42
46	*M. ruminantium*	4	BPS	50	44.8289	11.18
47	*M. ruminantium*	4	BPS	50	53.7022	13.40
48	*M. ruminantium*	4	BPS	50	53.5818	13.37
55	*M. ruminantium*	4	BPS	250	52.8235	13.18
56	*M. ruminantium*	4	BPS	250	52.8745	13.19
57	*M. ruminantium*	4	BPS	250	54.0834	13.49
64	*M. ruminantium*	4	propynoic acid	2	4.5767	1.14
65	*M. ruminantium*	4	propynoic acid	2	4.9978	1.25
66	*M. ruminantium*	4	propynoic acid	2	3.6932	0.92
73	*M. ruminantium*	4	propynoic acid	4	2.4213	0.60
74	*M. ruminantium*	4	propynoic acid	4	0.3907	0.10
75	*M. ruminantium*	4	propynoic acid	4	1.8737	0.47
82	*M. ruminantium*	4	ethyl 2-butynoate	4	0	0.00
84	*M. ruminantium*	4	ethyl 2-butynoate	4	0.2985	0.07
91	*M. ruminantium*	4	ethyl 2-butynoate	8	0	0.00
92	*M. ruminantium*	4	ethyl 2-butynoate	8	0	0.00
93	*M. ruminantium*	4	ethyl 2-butynoate	8	0	0.00
100	*M. ruminantium*	4	lumazine	100	0.3047	0.08
101	*M. ruminantium*	4	lumazine	100	0.2816	0.07
102	*M. ruminantium*	4	lumazine	100	0.3505	0.09
109	*M. ruminantium*	4	lumazine	50	1.6029	0.40
110	*M. ruminantium*	4	lumazine	50	1.9908	0.50
111	*M. ruminantium*	4	lumazine	50	1.9829	0.49
118	*M. ruminantium*	4	lumazine	25	25.0656	6.25
119	*M. ruminantium*	4	lumazine	25	18.1942	4.54
120	*M. ruminantium*	4	lumazine	25	7.1997	1.80
127	*M. ruminantium*	4	lumazine	1.2	0.9263	0.23
128	*M. ruminantium*	4	lumazine	1.2	1.0608	0.26
129	*M. ruminantium*	4	lumazine	1.2	1.3618	0.34
4	*M. mazei*	4	control	0	58.1063	13.97
5	*M. mazei*	4	control	0	56.1698	13.51
6	*M. mazei*	4	control	0	54.5838	13.12
13	*M. mazei*	4	BES	10	55.0705	13.24

Table A-42 (cont'd)

Tube	Species	Days	Treatment	Conc	Area	CH4, μmoles
14	*M. mazei*	4	BES	10	56.0643	13.48
15	*M. mazei*	4	BES	10	52.6799	12.67
22	*M. mazei*	4	BES	50	55.1997	13.27
32	*M. mazei*	4	BES	250	2.3774	0.57
33	*M. mazei*	4	BES	250	7.7439	1.86
40	*M. mazei*	4	BPS	10	53.2249	12.80
41	*M. mazei*	4	BPS	10	52.6379	12.66
42	*M. mazei*	4	BPS	10	59.255	14.25
49	*M. mazei*	4	BPS	50	53.2289	12.80
50	*M. mazei*	4	BPS	50	52.7157	12.68
51	*M. mazei*	4	BPS	50	53.2047	12.79
58	*M. mazei*	4	BPS	250	54.7244	13.16
59	*M. mazei*	4	BPS	250	54.9559	13.21
60	*M. mazei*	4	BPS	250	57.3977	13.80
67	*M. mazei*	4	propynoic acid	2	12.7036	3.05
68	*M. mazei*	4	propynoic acid	2	14.9247	3.59
69	*M. mazei*	4	propynoic acid	2	16.5176	3.97
76	*M. mazei*	4	propynoic acid	4	12.4457	2.99
77	*M. mazei*	4	propynoic acid	4	13.3465	3.21
78	*M. mazei*	4	propynoic acid	4	15.0857	3.63
85	*M. mazei*	4	ethyl 2-butynoate	4	53.2946	12.81
86	*M. mazei*	4	ethyl 2-butynoate	4	54.0697	13.00
87	*M. mazei*	4	ethyl 2-butynoate	4	53.8654	12.95
94	*M. mazei*	4	ethyl 2-butynoate	8	53.4904	12.86
95	*M. mazei*	4	ethyl 2-butynoate	8	56.9877	13.70
96	*M. mazei*	4	ethyl 2-butynoate	8	56.9475	13.69
103	*M. mazei*	4	lumazine	100	0.2188	0.05
104	*M. mazei*	4	lumazine	100	0.2589	0.06
105	*M. mazei*	4	lumazine	100	0.2941	0.07
112	*M. mazei*	4	lumazine	50	4.1121	0.99
113	*M. mazei*	4	lumazine	50	2.4709	0.59
114	*M. mazei*	4	lumazine	50	3.2069	0.77
121	*M. mazei*	4	lumazine	25	9.4995	2.28
122	*M. mazei*	4	lumazine	25	11.6769	2.81
123	*M. mazei*	4	lumazine	25	9.2108	2.21
130	*M. mazei*	4	lumazine	1.2	0.8735	0.21
131	*M. mazei*	4	lumazine	1.2	1.5954	0.38
132	*M. mazei*	4	lumazine	1.2	1.6077	0.39
7	*M. mobile*	4	control	0	45.2825	10.87
8	*M. mobile*	4	control	0	45.3007	10.88
9	*M. mobile*	4	control	0	37.7334	9.08
16	*M. mobile*	4	BES	10	44.4015	10.66
17	*M. mobile*	4	BES	10	44.8893	10.78
18	*M. mobile*	4	BES	10	45.4254	10.91
25	*M. mobile*	4	BES	50	46.0686	11.06
26	*M. mobile*	4	BES	50	44.7656	10.75
27	*M. mobile*	4	BES	50	44.9547	10.79

Table A-42 (cont'd)

Tube	Species	Days	Treatment	Conc	Area	CH4, μmoles
34	*M. mobile*	4	BES	250	0.5697	0.14
35	*M. mobile*	4	BES	250	0.7249	0.17
36	*M. mobile*	4	BES	250	0.6333	0.15
43	*M. mobile*	4	BPS	10	45.3587	10.89
54	*M. mobile*	4	BPS	50	46.1564	11.08
61	*M. mobile*	4	BPS	250	45.7709	10.99
62	*M. mobile*	4	BPS	250	45.056	10.82
63	*M. mobile*	4	BPS	250	44.9867	10.80
70	*M. mobile*	4	propynoic acid	2	4.6672	1.12
71	*M. mobile*	4	propynoic acid	2	4.8371	1.16
72	*M. mobile*	4	propynoic acid	2	4.992	1.20
79	*M. mobile*	4	propynoic acid	4	4.1809	1.00
80	*M. mobile*	4	propynoic acid	4	2.7309	0.66
81	*M. mobile*	4	propynoic acid	4	3.8537	0.93
88	*M. mobile*	4	ethyl 2-butynoate	4	45.2778	10.87
89	*M. mobile*	4	ethyl 2-butynoate	4	39.2063	9.44
90	*M. mobile*	4	ethyl 2-butynoate	4	45.6828	10.97
97	*M. mobile*	4	ethyl 2-butynoate	8	0.2201	0.05
98	*M. mobile*	4	ethyl 2-butynoate	8	0	0.00
99	*M. mobile*	4	ethyl 2-butynoate	8	0	0.00
106	*M. mobile*	4	lumazine	100	0	0.00
107	*M. mobile*	4	lumazine	100	0	0.00
108	*M. mobile*	4	lumazine	100	0	0.00
115	*M. mobile*	4	lumazine	50	1.2094	0.29
116	*M. mobile*	4	lumazine	50	1.4969	0.36
117	*M. mobile*	4	lumazine	50	0.2353	0.06
124	*M. mobile*	4	lumazine	25	4.5747	1.10
125	*M. mobile*	4	lumazine	25	11.4699	2.75
126	*M. mobile*	4	lumazine	25	17.9475	4.31
133	*M. mobile*	4	lumazine	1.2	0.8389	0.20
134	*M. mobile*	4	lumazine	1.2	0.257	0.06
135	*M. mobile*	4	lumazine	1.2	0.2342	0.06
1	*M. ruminantium*	6	control	0	53.1926	11.12
2	*M. ruminantium*	6	control	0	52.8989	11.05
3	*M. ruminantium*	6	control	0	53.1038	11.10
10	*M. ruminantium*	6	BES	10	36.0264	7.53
11	*M. ruminantium*	6	BES	10	53.3042	11.14
12	*M. ruminantium*	6	BES	10	47.92	10.01
19	*M. ruminantium*	6	BES	50	4.5598	0.95
20	*M. ruminantium*	6	BES	50	6.7496	1.41
21	*M. ruminantium*	6	BES	50	33.7218	7.05
28	*M. ruminantium*	6	BES	250	0	0.00
29	*M. ruminantium*	6	BES	250	0	0.00
30	*M. ruminantium*	6	BES	250	0	0.00

Table A-42 (cont'd)

Tube	Species	Days	Treatment	Conc	Area	CH4, µmoles
37	*M. ruminantium*	6	BPS	10	57.5412	12.02
8	*M. ruminantium*	6	BPS	10	59.5101	12.44
39	*M. ruminantium*	6	BPS	10	58.8977	12.31
46	*M. ruminantium*	6	BPS	50	49.7071	10.39
47	*M. ruminantium*	6	BPS	50	64.3493	13.45
48	*M. ruminantium*	6	BPS	50	59.5347	12.44
73	*M. ruminantium*	6	propynoic acid	4	3.1394	0.66
74	*M. ruminantium*	6	propynoic acid	4	0.4812	0.10
75	*M. ruminantium*	6	propynoic acid	4	2.4562	0.51
82	*M. ruminantium*	6	ethyl 2-butynoate	4	0	0.00
84	*M. ruminantium*	6	ethyl 2-butynoate	4	0.667	0.14
91	*M. ruminantium*	6	ethyl 2-butynoate	8	0.3035	0.06
92	*M. ruminantium*	6	ethyl 2-butynoate	8	0	0.00
93	*M. ruminantium*	6	ethyl 2-butynoate	8	0	0.00
100	*M. ruminantium*	6	lumazine	100	0.3982	0.08
101	*M. ruminantium*	6	lumazine	100	0.3057	0.06
102	*M. ruminantium*	6	lumazine	100	0.3864	0.08
109	*M. ruminantium*	6	lumazine	50	1.8802	0.39
110	*M. ruminantium*	6	lumazine	50	2.3708	0.50
111	*M. ruminantium*	6	lumazine	50	2.2893	0.48
118	*M. ruminantium*	6	lumazine	25	53.9594	11.28
119	*M. ruminantium*	6	lumazine	25	52.6593	11.00
120	*M. ruminantium*	6	lumazine	25	13.6578	2.85
127	*M. ruminantium*	6	lumazine	1.2	1.0263	0.21
128	*M. ruminantium*	6	lumazine	1.2	1.2244	0.26
129	*M. ruminantium*	6	lumazine	1.2	1.6568	0.35
4	*M. mazei*	6	control	0	49.8388	13.42
5	*M. mazei*	6	control	0	49.767	13.40
6	*M. mazei*	6	control	0	47.6735	12.84
13	*M. mazei*	6	BES	10	46.9728	12.65
14	*M. mazei*	6	BES	10	46.9852	12.66
15	*M. mazei*	6	BES	10	38.5586	10.39
22	*M. mazei*	6	BES	50	47.6245	12.83
23	*M. mazei*	6	BES	50	46.3932	12.50
24	*M. mazei*	6	BES	50	46.3357	12.48
31	*M. mazei*	6	BES	250	35.0087	9.43
32	*M. mazei*	6	BES	250	23.0265	6.20
33	*M. mazei*	6	BES	250	39.173	10.55
40	*M. mazei*	6	BPS	10	46.9075	12.63
41	*M. mazei*	6	BPS	10	46.2934	12.47
42	*M. mazei*	6	BPS	10	52.0599	14.02
49	*M. mazei*	6	BPS	50	47.0857	12.68

Table A-42 (cont'd)

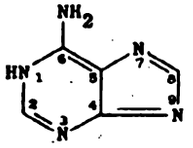
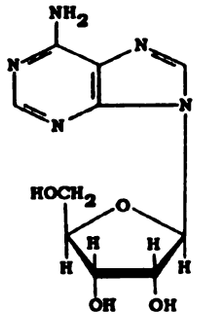
Tube	Species	Days	Treatment	Conc	Area	CH4, μmoles
50	*M. mazei*	6	BPS	50	45.5059	12.26
51	*M. mazei*	6	BPS	50	46.4766	12.52
58	*M. mazei*	6	BPS	250	47.8741	12.89
59	*M. mazei*	6	BPS	250	49.2702	13.27
60	*M. mazei*	6	BPS	250	50.0549	13.48
67	*M. mazei*	6	propynoic acid	2	11.005	2.96
68	*M. mazei*	6	propynoic acid	2	12.4005	3.34
69	*M. mazei*	6	propynoic acid	2	13.8477	3.73
76	*M. mazei*	6	propynoic acid	4	10.5017	2.83
77	*M. mazei*	6	propynoic acid	4	11.2987	3.04
78	*M. mazei*	6	propynoic acid	4	12.8494	3.46
85	*M. mazei*	6	ethyl 2-butynoate	4	46.3244	12.48
122	*M. mazei*	6	lumazine	25	16.8254	4.53
123	*M. mazei*	6	lumazine	25	11.075	2.98
130	*M. mazei*	6	lumazine	1.2	0.7116	0.19
131	*M. mazei*	6	lumazine	1.2	0.5504	0.15
132	*M. mazei*	6	lumazine	1.2	1.2557	0.34
7	*M. mobile*	6	control	0	40.0556	10.67
8	*M. mobile*	6	control	0	40.2887	10.74
9	*M. mobile*	6	control	0	33.1636	8.84
16	*M. mobile*	6	BES	10	39.9634	10.65
17	*M. mobile*	6	BES	10	39.6803	10.57
18	*M. mobile*	6	BES	10	40.41	10.77
25	*M. mobile*	6	BES	50	40.0242	10.67
26	*M. mobile*	6	BES	50	39.2076	10.45
27	*M. mobile*	6	BES	50	39.709	10.58
34	*M. mobile*	6	BES	250	1.3872	0.37
35	*M. mobile*	6	BES	250	3.2395	0.86
36	*M. mobile*	6	BES	250	1.8111	0.48
43	*M. mobile*	6	BPS	10	39.9844	10.65
44	*M. mobile*	6	BPS	10	39.7849	10.60
45	*M. mobile*	6	BPS	10	40.079	10.68
52	*M. mobile*	6	BPS	50	40.2914	10.74
53	*M. mobile*	6	BPS	50	40.5879	10.82
54	*M. mobile*	6	BPS	50	39.9171	10.64

Table A-42 (cont'd)

Tube	Species	Days	Treatment	Conc	Area	CH4, μmoles
61	*M. mobile*	6	BPS	250	39.6709	10.57
62	*M. mobile*	6	BPS	250	38.0327	10.13
63	*M. mobile*	6	BPS	250	40.3707	10.76
70	*M. mobile*	6	propynoic acid	2	4.0442	1.08
71	*M. mobile*	6	propynoic acid	2	3.9229	1.05
72	*M. mobile*	6	propynoic acid	2	4.1581	1.11
79	*M. mobile*	6	propynoic acid	4	3.683	0.98
80	*M. mobile*	6	propynoic acid	4	2.5434	0.68
81	*M. mobile*	6	propynoic acid	4	3.3839	0.90
88	*M. mobile*	6	ethyl 2-butynoate	4	39.9306	10.64
89	*M. mobile*	6	ethyl 2-butynoate	4	34.4073	9.17
90	*M. mobile*	6	ethyl 2-butynoate	4	39.7533	10.59
97	*M. mobile*	6	ethyl 2-butynoate	8	0	0.00
98	*M. mobile*	6	ethyl 2-butynoate	8	1.298	0.35
99	*M. mobile*	6	ethyl 2-butynoate	8	0	0.00

Chemical structures and physico-chemical properties of compounds studied

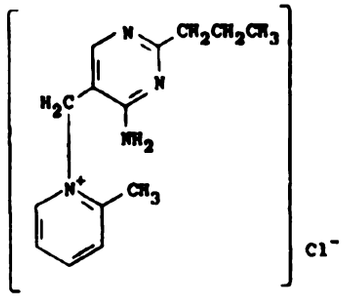
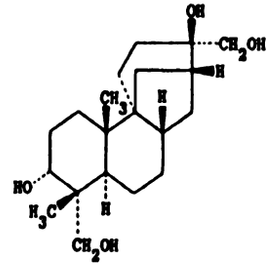
Table A-43 Chemical structures and physico-chemical properties of compounds studied

Name	Chemical formula	Molar mass (g/mol)	Density (g/mL)	Solubility in water (g/L)	Solubility in ether (g/L)	pK	LD_{50} (mg/kg)
Acetoacetate (Li salt)	CH_3COCH_2COOLi	108.0	NA[1]	completely miscible	NA	3.58	NA
Adenine		135.1	0.986 to 0.997	1.176 g/L	insoluble	-	745 (oral, rats)
Adenosine		267.2	NA	NA	NA	-	2,500 (intra peritoneal, mice)

[1]NA = not available.

Sources: CRC Handbook of Chemistry and Physics (1973), The Merck Index (1989), Acros catalog (1998-1999), Sigma catalog (2002-2003), Beilstein database (2002).

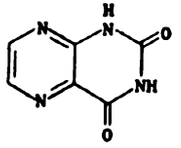
Table A-43 (cont'd)

Name	Chemical formula	Molar mass (g/mol)	Density (g/mL)	Solubility in water (g/L)	Solubility in ether (g/L)	pK	LD_{50} (mg/kg)
Amprolium		278.8	NA[1]	completely miscible	NA	-	6,170 (oral, mice)
Aphidicolin		338.5	NA	NA	NA	-	NA
2-Bromoethanesulfonate (Na salt)	$BrCH_2CH_2SO_3Na$	211.0	NA	NA	NA	NA	NA
3-Bromopropanesulfonate (Na salt)	$BrCH_2CH_2\ CH_2SO_3Na$	225.0	NA	NA	NA	NA	NA
Br-pyruvic acid	$BrCH_2COCOOH$	167.0	NA[1]	NA	NA	NA	72 (intra peritoneal, mice)
3-Butenoic acid	$H_2C{=}CHCH_2COOH$	86.1	1.013	soluble	completely miscible	4.34	NA

[1]NA = not available.

Sources: CRC Handbook of Chemistry and Physics (1973), The Merck Index (1989), Acros catalog (1998-1999), Sigma catalog (2002-2003), Beilstein database (2002).

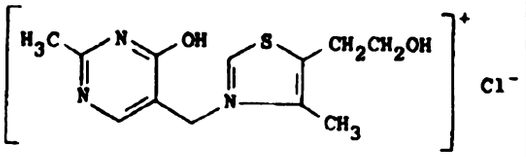
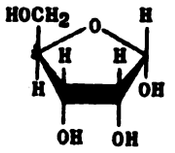
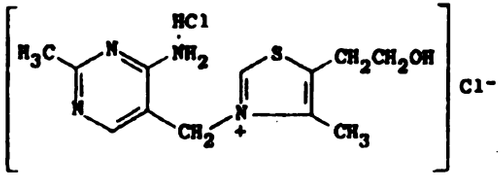
Table A-43 (cont'd)

Name	Chemical formula	Molar mass (g/mol)	Density (g/mL)	Solubility in water (g/L)	Solubility in ether (g/L)	pK	LD_{50} (mg/kg)
2-Butynoic acid	$CH_3C{\equiv}CCOOH$	84.1	0.964	soluble	soluble	NA[1]	NA
Crotonic acid	$CH_3CH{=}CHCOOH$	86.1	1.027	76.1	soluble	4.70	1,000 (oral, rats) 4,800 (oral, mice)
Ethyl 2-butynoate	$CH_3C{\equiv}CCOOCH_2CH_3$	112.1	0.962	Insoluble	sompletely miscible	-	NA
Fluoropyruvate (Na salt)	$CH_2FCOCOONa$	128	NA	NA	NA	NA	125 (intra peritoneal, mice)
Hexadecatrienoic acid	$CH_3CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_4COOH$	236	0.9	NA	NA	NA	NA
ß-Hydroxybutyrate (Na salt)	$CH_3CHOHCH_2COONa$	126.1	NA	sompletely miscible	sompletely miscible	4.41	1,106 (intra venous, rat) 860 (intra peritoneal, mice) 724 (intra peritoneal, cat)
Hydroxypyruvic acid	$CH_2OHCOCOOH$	104.1	NA	NA	NA	NA	NA
Lumazine		164.1	NA	1.25 g/L	NA	-	> 400 (intra peritoneal, rats)
Oxaloacetic acid	$HOOCCH_2COCOOH$	132.1	NA	soluble	soluble	2.56	NA

[1]NA = not available.

Sources: CRC Handbook of Chemistry and Physics (1973), The Merck Index (1989), Acros catalog (1998-1999), Sigma catalog (2002-2003), Beilstein database (2002).

Table A-43 (cont'd)

Name	Chemical formula	Molar mass (g/mol)	Density (g/mL)	Solubility in water (g/L)	Solubility in ether (g/L)	pK	LD_{50} (mg/kg)
Oxythiamine		301.8	NA[1]	NA	NA	-	1,430 (sub cutaneous, mice) 160 (sub cutaneous, rat)
Propynoic acid	CH≡CCOOH	70.1	1.138	completely miscible	completely miscible	NA	50 (oral, mice)
Pyruvate (Na salt)	$CH_3COCOONa$	110.0	1.267	completely miscible	soluble	2.49	NA
D-Ribose		150.1	NA	soluble	NA	-	NA
Thiamin hydrochloride		337.3	NA	1000 g/L	insoluble	-	89,200 (intra venous., mice) 8,224,000 (oral, mice)

[1]NA = not available.

Sources: CRC Handbook of Chemistry and Physics (1973), The Merck Index (1989), Acros catalog (1998-1999), Sigma catalog (2002-2003), Beilstein database (2002).

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