Homoacetogenesis as an alternative hydrogen sink in the rumen

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Abstract

Ruminant livestock contribute significantly to global greenhouse gas emissions. This is due to microorganisms, known as methanogens that generate methane from hydrogen and carbon dioxide during feed fermentation in the rumen. Mitigation strategies are being developed to reduce methane emissions from ruminants. However, inhibiting methane production may cause accumulation of unused hydrogen in the rumen, which may slow down rumen fermentation and affect animal productivity. Homoacetogens, microbes known to reside in the rumen, can use hydrogen and carbon dioxide to form acetate. Homoacetogens could take over the role of ruminal hydrogen disposal following inhibition of methanogens. The aims of this study were to quantify the involvement of alternative hydrogen utilisers, such as homoacetogens, in hydrogen or electron utilisation. Chemical compounds were screened to identify specific inhibitors of methanogens (BES, acetylene), and both methanogens and homoacetogens (chloroform). Homoacetogenesis was measured via incorporation of $^{13}CO_2$ into ^{13}C-acetate using a short-term *in vitro* assay. This short-term *in vitro* assay measured and confirmed the occurrence of homoacetogenesis in sheep rumen fluid, and it accounted for 1.67% of electron utilisation in fresh rumen fluid. Homoacetogenesis increased in the assay when BES was added, suggesting homoacetogens could increase their activity in the absence of methanogens. Homoacetogenesis decreased with the addition of chloroform, which is known to partially inhibit homoacetogens. Methane formation was inhibited by acetylene in an *in vitro* serial batch fermentation inoculated with sheep rumen fluid. Homoacetogenesis did not increase, but the homoacetogens were able to grow and maintain themselves as the rumen material was repeatedly diluted and supplemented with fresh feed. Their activity accounted for 2.32% of electron utilisation. To study their significance in the rumen, methane formation was inhibited in sheep using acetylene. Homoacetogenesis increased and accounted for 6.53% of electron utilisation. However, propionate appeared to be the major electron sink (58-88%) in the absence of methanogenesis both *in vitro* and *in vivo*. In the future, knowledge of these hydrogen-utilising microorganisms could be used to divert hydrogen or electrons into more beneficial end-products, leading to the transition from a normal methane-producing rumen to an equally or even more productive low methane one.

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Abbreviations

With a few exceptions (to avoid confusion), standard SI units are not defined here.

ACS	acetyl-CoA synthetase
ATP	adenosine triphosphate
atm	atmosphere
BES	2-bromoethanesulfonate
bp	base pair
BSA	bovine serum albumin
CH_4	methane
$CHCl_3$	chloroform
CoM	coenzyme M
CO_2	carbon dioxide
CODH	carbon monoxide dehydrogenase
Conc.	concentration, concentrated
dNTP	deoxynucleotide triphosphate
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FTHFS	formyltetrahydrofolate synthetase
g	gravity
$\Delta G°'$	Gibb's (free) energy change
GC	gas chromatography
GC-FID	gas chromatography with flame ionisation detector
GC-MS	gas chromatography mass spectrometry
GC-IRMS	gas chromatography with isotope ratio mass spectrometry
2GenRFV	GCXAL-CPY-rumen fluid-vitamin mix with double substrate concentrations
GHG	greenhouse gases
GP	general purpose diet
H_2	hydrogen
HPLC	high performance liquid chromatography
HMM	hidden Markov model

HMMER	profile hidden Markov model software
IPTG	isopropyl β-D-1-thiogalactopyranoside
kJ/mol	kilojoules per mole
K_s	half saturation constant (also referred to as Monod's constant)
LB	Luria-Bertani
Ltd.	limited
M	molar
min	minutes
m/z	mass-to-charge ratio
N_2	nitrogen
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAD(P)H	NADH or NADPH
$NaHCO_3$	sodium hydrogen carbonate
NoSubRFV	rumen fluid vitamin mix with no added growth substrates
PCR	polymerase chain reaction
psi	pounds per square inch
qPCR	quantitative real-time polymerase chain reaction
QIIME	quantitative insights into microbial ecology
RCC	Rumen Cluster C
RF	rumen fluid
RM02	rumen medium number 2
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S	dissolved substrate concentration
S_{min}	minimum threshold substrate concentration
SPME	solid-phase micro extraction
SD	standard deviation
SEM	standard error of the mean
TAE	tris-acetate-EDTA
U/µl	units/microlitre
v/v	volume per volume
V	relative rate of metabolism
V_{max}	maximum rate of metabolism

VFA	volatile fatty acid(s)
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Mathematical abbreviations

A	amount of acetate produced
A_f	amount of acetate formed from fermentation
A_{ha}'	amount of acetate produced from homoacetogenesis (VFA inter-conversion uncorrected)
A_{ha}	amount of acetate (unlabelled and labelled) produced via homoacetogenesis (VFA inter-conversion corrected)
A^*	$^{13}C_1$-acetate
A^{**}	$^{13}C_2$-acetate
^{13}A	amount of excess labelled acetate
$^{13}A_{ha}$	amount of ^{13}C-labelled acetate produced via homoacetogenesis
B	amount of butyrate produced
B_f	amount of butyrate formed from fermentation
B_{hb}'	amount of butyrate produced from homobutyrogenesis (VFA inter-conversion uncorrected)
B_{hb}	amount of butyrate (unlabelled and labelled) produced via homobutyrogenesis (VFA inter-conversion corrected)
B^*	$^{13}C_1$-butyrate
B^{**}	$^{13}C_2$-butyrate
B^{***}	$^{13}C_3$-butyrate
B^{****}	$^{13}C_4$-butyrate
^{13}B	amount of excess labelled butyrate
$^{13}B_{hb}$	amount of ^{13}C-labelled butyrate produced via homobutyrogenesis
f_{ab}	fractional amount of ^{13}C-acetate converted to ^{13}C-butyrate
f_{ap}	fractional amount of ^{13}C-acetate converted to ^{13}C-propionate
f_{ba}	fractional amount of ^{13}C-butyrate converted to ^{13}C-acetate
f_{bp}	fractional amount of ^{13}C-butyrate converted to ^{13}C-propionate
f_{pa}	fractional amount of ^{13}C-propionate converted to ^{13}C-acetate
f_{pb}	fractional amount of ^{13}C-propionate converted to ^{13}C-butyrate
H_2	amount of hydrogen formed from fermentation
$2H$	two reduced protons, representing two electrons
m/z	mass-to-charge ratio
M	amount of methane formed from fermentation

MPE_i	mole percent excess for any species containing *i* labelled carbons
MPE_0	mole percent excess for any species containing no labelled carbon
MPE_1	mole percent excess for any species containing one labelled carbon
MPE_2	mole percent excess for any species containing two labelled carbons
MPE_3	mole percent excess for any species containing three labelled carbons
MPE_4	mole percent excess for any species containing four labelled carbons
P	amount of propionate produced
P_f	amount of propionate formed from fermentation
P^*	$^{13}C_1$-propionate
P^{**}	$^{13}C_2$-propionate
P^{***}	$^{13}C_3$-propionate
^{13}P	amount of excess labelled propionate
rA_{Lferm}	ratio of $^{13}C/^{12}C$ in acetate in fermentations with $NaH^{13}CO_3$
rA_{Uferm}	ratio of $^{13}C/^{12}C$ in acetate in fermentations with unlabelled $NaHCO_3$
rB_{Lferm}	ratio of $^{13}C/^{12}C$ in butyrate in fermentations with $NaH^{13}CO_3$
rB_{Uferm}	ratio of $^{13}C/^{12}C$ in butyrate in fermentations with unlabelled $NaHCO_3$
$rCO_{2\ Lferm}$	ratio of $^{13}C/^{12}C$ in CO_2 in fermentations with $NaH^{13}CO_3$
$rCO_{2\ Uferm}$	ratio of $^{13}C/^{12}C$ in CO_2 in fermentations with unlabelled $NaHCO_3$
rP_{Lferm}	ratio of $^{13}C/^{12}C$ in propionate in fermentations with $NaH^{13}CO_3$
rP_{Uferm}	ratio of $^{13}C/^{12}C$ in propionate in fermentations with unlabelled $NaHCO_3$
$r^{13}CO_2$	ratio of excess of dissolved $^{13}CO_2$ relative to control without $NaH^{13}CO_3$
R_1	selected ion peak area ratios for ions m/z 108:107 for sample
R_{10}	selected ion peak area ratios for ions m/z 108:107 for control
R_2	selected ion peak area ratios for ions m/z 109:107 for sample
R_{20}	selected ion peak area ratios for ions m/z 109:107 for control
R_3	selected ion peak area ratios for ions m/z 110:107 for sample
R_{30}	selected ion peak area ratios for ions m/z 110:107 for control
R_4	selected ion peak area ratios for ions m/z 111:107 for sample
R_{40}	selected ion peak area ratios for ions m/z 111:107 for control
V_f	amount of valerate formed from fermentation
X_a	fraction of acetate coming from CO_2
X_b	fraction of butyrate formed from homobutyrogenesis

Chapter 1

Literature review

1.1 Global and New Zealand greenhouse gas emissions

Global climate change is characterised by increases in the earth's temperature, resulting in an average combined global land and ocean surface temperature increase of 0.85 °C (0.65 to 1.06 °C) from 1880 to 2012 (IPCC 2014a). This represents a major global issue, as increases in global temperatures can lead to melting of ice and glaciers, which contributes to rise in sea levels, changes in precipitation in low and high rainfall areas, which in turn has effects on distribution of wet and desert areas, and can increase the prevalence of human and animal diseases (Moss *et al.* 2000, Gerstengarbe & Werner 2008). Furthermore, it appears that the increase in the global surface temperature is likely to exceed 1.5 °C by the end of 21st century (2081-2100; IPCC 2014a). A rise in greenhouse gases (GHG) is thought to have caused climate change, especially as atmospheric GHG concentrations have increased significantly between 1970 to 2010, despite an increasing number of climate change mitigation policies and strategies (IPCC 2014). The anthropogenic sources of GHG emissions differ between countries, but mostly relate to energy, agriculture, forestry and other land use, industry, transport, and building sectors (Figure 1.1a; IPCC 2014a). The three major anthropogenic greenhouse gases are carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O; Steinfeld *et al.* 2006). Methane has a 100-year global warming potential of 28 (IPCC 2014b), even though it has a much shorter atmospheric lifetime (Ripple *et al.* 2014). Methane emissions from enteric fermentation represents 27% of global anthropogenic methane emissions (Figure 1.1b; IPCC 2014b).

Agriculture is central to New Zealand's economy, and it is a major contributor to New Zealand's export earnings. Dairy and meat exports accounted for 41% of the total value of exports in 2014 (Ministry for the Environment & Statistics New Zealand, 2015). In New Zealand, 47% of total GHG emissions are a direct consequence of agriculture, and methane represents 32% of total anthropogenic GHG emissions, and is nearly all produced from ruminant enteric fermentation (Ministry for the Environment, 2014).

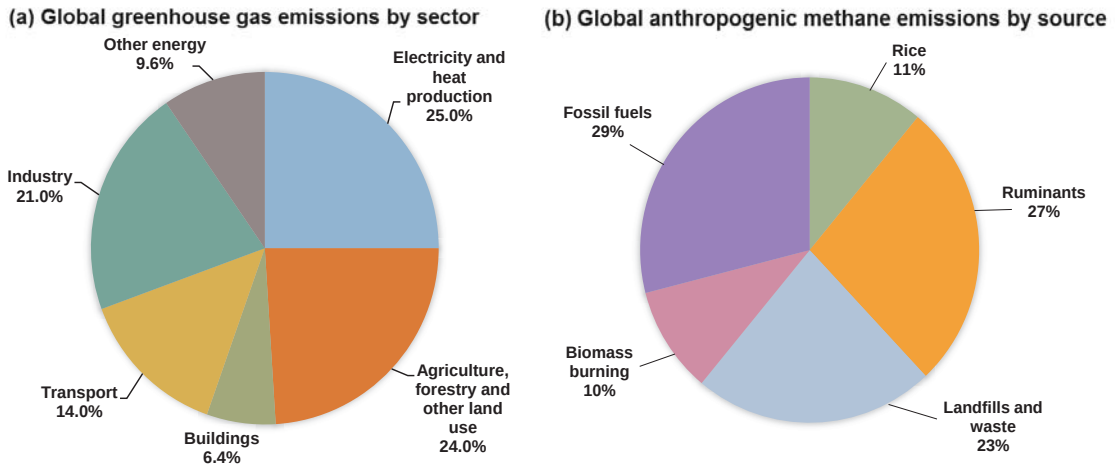

Figure 1.1 (a) Total anthropogenic greenhouse gas emissions by economic sector in 2010 (IPCC 2014a). (b) Estimated global anthropogenic methane emissions by source in 2000-2009 (IPCC 2014b).

1.2 Rumen function

Ruminant livestock such as sheep, cattle, domesticated deer and goats play a significant role in agriculture. They are capable of converting human indigestible feeds into human-edible products (Oltjen & Beckett 1996). Ruminants provide nations worldwide with products such as meat, dairy products, wool and leather.

1.2.1 Ruminant digestive system and microbial feed digestion

Ruminants differ from non-ruminants in that they have a modified foregut and stomach with four compartments, namely the rumen, reticulum, omasum and abomasum (Figure 1.2). The rumen plays a major role in feed fermentation in ruminants. The rumen is a large, relative to body size, anaerobic fermentation chamber, with a pH between 5.6 and 6.7, and a temperature of 39 °C, which allows the growth of microbes, facilitating the digestion and fermentation of cellulosic plant material (Hungate 1966, Kolver & de Veth 2002). The lack of oxygen is important, as this means that there can only be a partial microbial oxidation, and the fermentation products are available for use by the ruminant host.

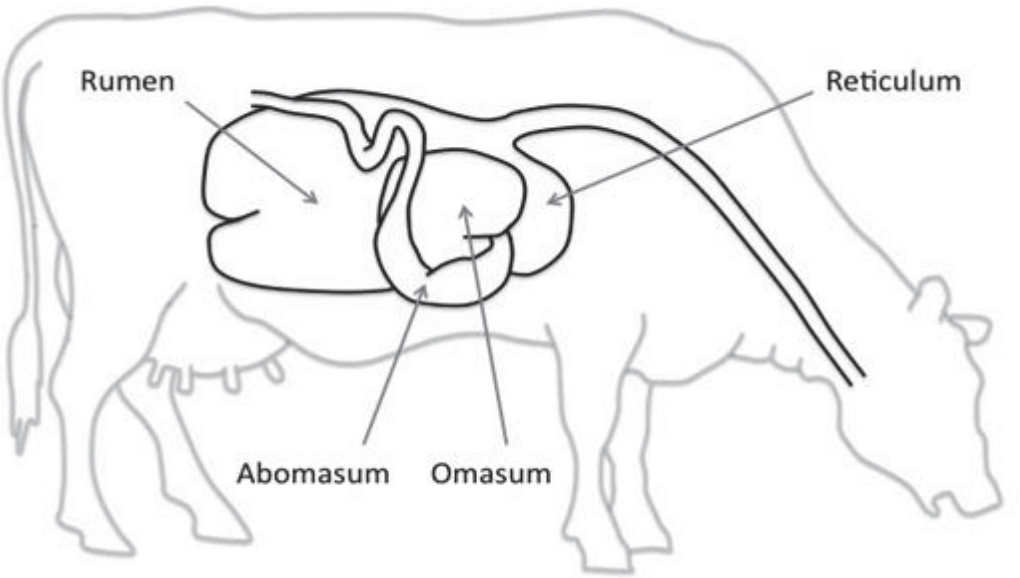

Figure 1.2 Foregut showing the rumen and its compartments in relation to the stomach (abomasum) of a cow. Drawn by Peter Janssen, and used with permission.

Feed ingested by the ruminants consists of complex structural polysaccharides, starch, simpler sugars, lipids, proteins, lignin and minerals. The structural polysaccharides can be categorised into cellulose, hemicellulose and pectin (Wang & McAllister 2002). This feed enters the rumen, which harbours bacteria, protozoa and fungi. They enzymatically digest feed to simpler components, which in turn are fermented to end-products such as volatile fatty acids (VFA; France & Dijkstra 2005), that provide 70% of the animals metabolisable energy requirements (Bergman 1990). The VFA formed include acetate (approx. 65%), propionate (approx. 20%) and butyrate (approx. 15%), which form the major part of acids in the rumen (Miller 1979; Figure 1.3). The exact ratios of the VFA depends on the feed and the pathways of electron disposal active in the rumen microbes. Additionally, gases such as CO_2, hydrogen (H_2) and methane are also formed (Kamra 2005).

Hydrogen is used by methanogenic archaea and other hydrogen-utilising bacteria in the rumen, and is regarded as the main "currency" of rumen fermentation (Czerkawski 1986). The dissolved hydrogen concentration in the rumen lies in the range of 0.1 to 50 µM (Janssen 2010). Ruminal hydrogen is produced by hydrogenase enzymes acting on reduced cofactors, such as ferredoxin, nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH), which are formed during glucose fermentation (Hegarty & Gerdes 1999). These

reduced cofactors transfer electrons to hydrogenase enzymes, which then reduce $2H^+$ to hydrogen. During rumen fermentation, hydrogen is produced by some rumen microbial species and used by the others, and this is referred to as "interspecies H_2 transfer" (Krause *et al.* 2013). The symbiosis between feed-fermenting hydrogen-producing bacteria on the one hand, and methanogenic archaea and other hydrogen-utilising bacteria on the other, results in enhanced feed digestion (Lascano & Cárdenas 2010). One such example of interspecies hydrogen transfer, and one of the very first studied, is the ethanol-oxidising microbe called the "S organism" and *Methanobacterium bryantii*. The S organism produces hydrogen, which is used by *Methanobacterium bryantii*, which in turn removes hydrogen, which otherwise would inhibit the growth of the S organism (Bryant *et al.* 1967, Reddy *et al.* 1972). The S organism is an example of an obligate requirement for interspecies hydrogen transfer. In the absence of a hydrogen using partner, the S organism can barely grow. Another example of interspecies hydrogen transfer is the symbiosis between protozoa and methanogens. Protozoa produce hydrogen in their specialised oragnelles called hydrogenosomes (Embley *et al.* 2003). This hydrogen is used by methanogens present either inside or outside the protozoal cells, and in return benefits protozoa by hydrogen removal which otherwise inhibits their metabolism (Sharp *et al*. 1998, Belanche *et al*. 2014). Anaerobic fungi are also known to have symbiotic relationships with methanogens based on interspecies hydrogen transfer (Bauchop & Mountfort 1981, Joblin *et al*. 2002). In the rumen, the interspecies hydrogen transfer is mainly facultative. The hydrogen producers can grow in the absence of hydrogen users, but the presence of the latter can enhance growth of the former, or change the fermentation patterns so that more hydrogen is formed from each molecule of substrate fermented and when the hydrogen is used, the low concentrations of the dissolved gas are maintained (Janssen 2010). Methane formation appears to be the major pathway of hydrogen utilisation in the rumen, as hydrogen formed by fermentative micro-organisms (bacteria, fungi and protozoa) is transferred to methanogens, which then leads to re-oxidation of reduced cofactors (Wolin *et al.* 1997).

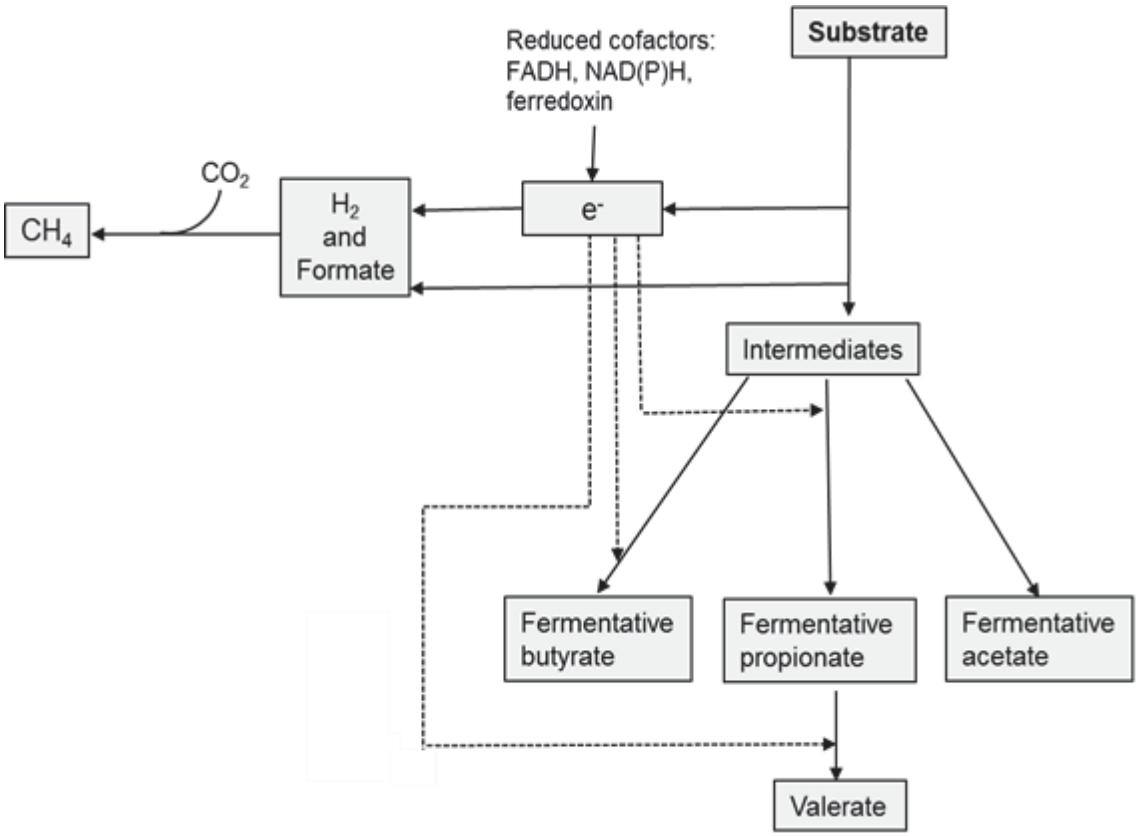

Figure 1.3 Major substrate fermentation pathways in the rumen (unbalanced). Methane is formed from carbon dioxide (CO_2) and hydrogen (H_2). Acetate, propionate and butyrate are the major VFAs produced. Dashed arrows (- - -►) show that electrons (e^-) can also be used in formation of propionate, butyrate and valerate.

1.2.2 Rumen microbes

The rumen is the habitat for a diverse and complex community of microbes including fungi (10^3-10^6 cells per ml), protozoa (10^4-10^6 cells per ml), bacteriophages (10^9-10^{10} particles per ml), bacteria (10^{10}-10^{11} cells per ml) and methanogenic archaea (10^7-10^9 cells per ml), which are actively involved in the digestion of feed material (Klieve & Swain 1993, Lin *et al.* 1997, Karnati *et al.* 2003, Griffith *et al.* 2010, Hook *et al.* 2010, Wright & Klieve 2011, Morgavi *et al.* 2012). Fungi are the initial colonizers of plant fibre in the rumen, and help degrade polysaccharides such as cellulose and starch (Williams & Orpin 1987, Heath 1988). Protozoa mostly adhere to feed particles and help with fibre degradation, and are also known to digest bacteria as a protein source (Dehority 2004). Bacteriophages cause the lysis of bacteria in the rumen, which can reduce the efficiency of feed conversion in ruminants (Klieve & Swain 1993, Hespell *et al.* 1997). Bacteria form the majority of organisms present in the rumen based on number, cell mass and small subunit ribosomal RNA (Lin *et al.* 1997). The seven most abundant rumen bacterial

groups globally are from the genera *Prevotella*, *Butyrivibrio* and *Ruminococcus,* unclassified members of the families *Lachnospiraceae* and *Ruminococcaceae*, and unclassified members of the orders *Bacteroidales* and *Clostridiales* (Henderson *et al.* 2015). These fermentative bacteria produce VFA as fermentation end-products, which are absorbed across the rumen wall. Rumen archaeal communities are comprised of methanogens that belong to the phylum *Euryarchaeota* and these are responsible for methane production (Balch *et al.* 1979). *Methanobrevibacter gottschalkii* (48.1%), *Methanobrevibacter ruminantium* (24.6%), *Methanosphaera* sp. ISO3-F5 (6.2%), *Methanomassiliicoccaceae* Group 12 sp. ISO4-H5 (6.2%) and *Methanomassiliicoccaceae* Group 10 sp. (3.1%) are the most dominant archaeal groups in the rumen, comprising more than 88.2% of archaeal community (Henderson *et al.* 2015). However, there are large variations in the abundance of each methanogen species driven largely by diet, but also animal-to-animal variation.

1.2.3 Ruminant methane production

Methanogens can use different substrates to form methane, including hydrogen, formate, methyl compounds and acetate (Thauer *et al.* 1977). Methanogens can be classified depending on their ability to use different energy sources (Rouviere & Wolfe 1988). Methanogens that use methyl compounds such as methanol formed from pectin breakdown or dietary mono, di- and tri-methylamines to form methane are referred to as methylotrophs (Czerkawski & Breckenridge 1972, Neill *et al.* 1978). In the rumen true methylotrophs are rare (Henderson et al. 2015). Instead, most rumen methylotrophs have an obligate requirement for hydrogen as a co-substrate, such as members of the genus *Methanosphaera* and the order *Methanomassiliicoccales* (Henderson et al. 2015). Acetate can also be used by methanogens to form methane, and these methanogens are known as acetoclastic methanogens (Ferry 1992). However, other than under a few exceptional dietary conditions, methane is not formed from acetate in the rumen (Rowe *et al.* 1979). Methanogens that convert hydrogen and carbon dioxide, and or formate to methane are referred to as hydrogenotrophs. Henderson et al. (2015) estimated that nearly 80% of rumen methanogens are hydrogenotrophs. Hungate calculated that 18% of ruminant derived methane originates from formate (Hungate *et al.* 1970). Therefore, hydrogen is regarded as the most important substrate or intermediate in rumen methane production (Hungate 1967).

Methane production is directly proportional to the rate of hydrogen uptake in the rumen fluid (Czerkawski 1972). The rate of methane formation is highest immediately after feeding, as the partial pressure of hydrogen is also high due to the increased rate of feed fermentation (Smolenski & Robinson 1988, Johnson *et al.* 1994). Removal of hydrogen by methanogens in the rumen allows microbes involved in fermentation to function optimally and also helps with complete substrate fermentation. This is because the hydrogen has a feed-back effect that is postulated to slow the rate of feed fermentation (McAllister & Newbold 2008). Approximately 87% of enteric methane is formed in the rumen, and 13% is produced from fermentation in the large intestine (Lockyer & Jarvis 1995, Lassey *et al.* 1997). The methane released represents a loss of 2-12% of the feed's-derived energy for the animal, dependent on the feed (Johnson & Johnson 1995). Methane is lost from the animal, mainly through eructation, and is not degraded by any microbes that are able to maintain themselves in the rumen. Methane emissions from ruminants vary and are known to depend on feed intake, and genetic background of the animal (Johnson & Ward 1996, Herd *et al.* 2002). As mentioned earlier, methane released by the animal into the atmosphere also contributes to GHG emissions (Rossi *et al.* 2001; see also Section 1.1). The impacts methane formation has on animal productivity and the environment mean that there is significant global interest in finding sustainable ways to mitigate ruminant methane emissions.

1.3 Strategies to reduce methane production and consequences of inhibition of methane production

Reducing methane emissions from ruminants has been extensively investigated over the past forty years. Strategies used can be broadly classified as 1) dietary manipulation, 2) alternative hydrogen and electron sinks, and 3) directly inhibiting methanogens (Figure 1.4).

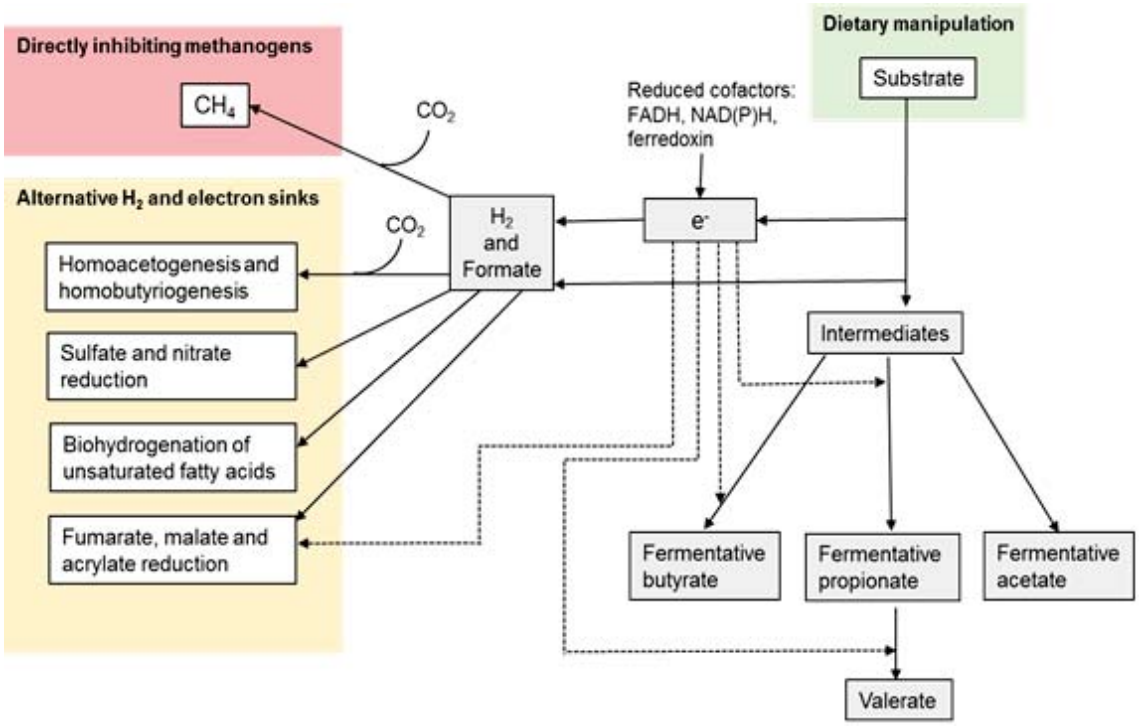

Figure 1.4 Strategies to redirect rumen fermentation (unbalanced reactions) towards less methane being produced. Dashed arrows (- - -▶) show that electrons (e^-) can also be used in formation of propionate, butyrate, and valerate, and can be diverted towards other electron acceptors present in the rumen by addition of additives (e.g., fumarate, malate). The other hydrogen utilising pathways are nitrate and sulfate-reduction pathways, biohydrogenation of unsaturated fatty acids, and homoacetogenesis and homobutyrogenesis (present in the rumen).

1.3.1 Dietary manipulation

The aim of dietary manipulation is to formulate or select a feed that results in less hydrogen being formed, thereby resulting in less methane formation. The amount of methane formed by ruminants largely depends on the type and amount of feed ingested, its rumen digestibility and ruminal passage rate, and the amount of hydrogen formed from it (Kennedy & Milligan 1978, Johnson & Johnson 1995, Pelchen & Peters 1998, Hook *et al.* 2010, Janssen 2010, Kumar *et al.* 2014). This is because different diets provide different substrates for microbial fermentation and with different digestibility, which can lead to changes in VFA production profile and methane (Murphy *et al.* 1982, Friggens *et al.* 1998). The forage to concentrate ratio of diets appears to have a significant impact on methane emissions from animals (Whitelaw *et al.* 1984, Benchaar *et al.* 2001, Beauchemin & McGinn 2005). For example, high forage diets usually result in more methane being formed per unit of dry matter or organic matter digested than do high grain diets (Harper *et al.* 1999, Beauchemin & McGinn 2005). Forages usually have higher

contents of structural carbohydrates, which are degraded slowly as compared to starches, whereas grains usually contains less structural carbohydrates and more rapidly degradable starches (Hungate 1966, Sutton 1971). Methane production was reduced by 56% when a diet containing 45% starch was fed, instead of a diet containing 30% starch (Patra 2012). This is likely due to starch significantly affecting ruminal pH and microbial populations. It has also been observed that increasing the rate of digestion and passage of the feed also results in less methane formation. Therefore, forages which are finely chopped, treated or pelleted also result in less methane production (Blaxter & Graham 1956, Moss *et al.* 1994, Hironaka *et al.* 1996). Diets containing chicory are also readily degraded, resulting in a high rate of digestion and low methane emissions than animals fed ryegrass (Swainson *et al.* 2008). Feeding brassica forages such as rape and swedes to sheep resulted in methane yields 23% and 25% lower, respectively, than from ryegrass, which might be due to the high digestibility of brassicas (Sun *et al.* 2012). Increases in passage rate of feed from the rumen leading to decreases in methane formation has reported previously (Kennedy & Milligan 1978, Pinares-Patiño *et al.* 2003). This decrease in methane formation with increased passage rate or increased digestibility can be attributed to fermentation pathways that lead to formation of more propionate, less hydrogen and therefore, less methane (Janssen 2010).

Addition of lipids, fatty acids, and oils to the diet has also been shown to mitigate methane. Oils rich in medium-chain fatty acids (C_8-C_{16}) have been found to suppress methane formation (Dong *et al.* 1997, Dohme *et al.* 2000). Supplementation of fish oil *in vitro* decreased methane formation by 80% (Fievez *et al.* 2003) and coconut oil reduced methane production by 25% *in vivo* (Machmüller *et al.* 2000). The toxic effects of canola oil and coconut oil on protozoa resulted in reduction in methane *in vitro* due to a decrease in hydrogen produced by protozoa, which was therefore not available for use by methanogens (Dong *et al.* 1997, Machmüller *et al.* 1998, Dohme *et al.* 2001).

1.3.2 Alternative hydrogen or electron sinks

Feed can be supplemented with additives that divert the reduced cofactors away from reducing H^+ ions (to form hydrogen) to reducing other reducible species termed "electron sinks", so resulting in less hydrogen and ultimately less methane being formed in the rumen. This can also divert some of the hydrogen formed into pathways that do not result in methane formation.

Fumarate, malate and acrylate reduction to increase propionate production

Propionate is one of the major products formed during rumen fermentation (Lindsay 1978). Propionate formation in the rumen can occur via two pathways, the non-randomising (acrylate) pathway associated with no ATP generation or the randomising (succinate) pathway associated with ATP generation (Thauer *et al.* 1977, Russell & Wallace 1997, Ungerfeld & Kohn 2006). Acrylate, as acryl-CoA, is an intermediate in the non-randomising pathway, and is reduced to propionate. The randomising pathway is the major propionate synthesis pathway from glucose (Baldwin *et al.* 1963). Fumarate and malate are intermediates in this pathway, which are further reduced to succinate, which is decarboxylated to propionate. In several *in vitro* and *in vivo* ruminal studies, addition of fumarate, malate or acrylate resulted in reduced methane production and increased propionate formation (Martin & Streeter 1995, Asanuma *et al.* 1999, Carro *et al.* 1999, Iwamoto *et al.* 1999, Lopez *et al.* 1999, Bayaru *et al.* 2001). It has been reported that fumarate and malate result in the formation of varying proportions of acetate and propionate in mixed rumen cultures, and this might have an impact on hydrogen availability to methanogens (Ungerfeld & Kohn 2006, Ungerfeld *et al.* 2007). The amount of extra propionate formed due to either of the two pathways after inhibition of methane formation is however unknown (Ungerfeld 2015).

Nitrate and sulfate reduction

The nitrate reduction pathway is thermodynamically more favourable than methanogenesis and it utilises four moles of H_2, which could reduce methane production by a mole in the rumen (van Zijderveld *et al.* 2010). Following the addition of nitrate *in vitro* (Lewis 1951, Allison & Reddy 1984, Guo *et al.* 2009) and *in vivo* (Alaboudi & Jones 1985, Takahashi *et al.* 1998, Sar *et al.* 2004), methane production was reduced. Formation of nitrite from nitrate is much faster than nitrite reduction to ammonia (Iwamoto *et al.* 1999) and accumulation of nitrite is toxic for the animal (Lewis 1951, Morris *et al.* 1958, Ozmen *et al.* 2005). This limits the suitability of nitrate as an alternative hydrogen sink. Interactions between nitrate and sulfur-oxidising bacteria have been reported to occur in ruminants (Lewis 1954, Anderson 1956). For example, sulfide formed during sulfate reduction can act as an electron donor during the reduction of nitrite to ammonia by nitrate-reducing, sulfur-oxidizing bacteria (Hubert & Voordouw 2007). Sulfate-reducing bacteria (SRB) are hydrogen-using bacteria that are present in the rumen (Stewart *et al.* 1997). SRB are present in different numbers in different animals such as in sheep

approximately 2 x 10^6/ml and cattle 8.2 x 10^5/ml (Morvan *et al.* 1996). *Desulfovibrio* sp. is the most commonly found and abundant genus, and is responsible for most of the sulfate-reduction in ruminants (Howard & Hungate 1976, Lin *et al.* 1997). They are, however, not particularly abundant in the rumen (Henderson et al. 2015). SRB mostly use nitro-compounds, nitrate and sulfate as electron acceptors, and are considered to be competitors of methanogens for hydrogen (Gibson *et al.* 1993). However, due to the limited availability of oxidised sulfur compounds in the animal diet, SRB do not represent a major hydrogen sink (Zinder 1993). Addition of sulfur or cysteine to the diet can aid in the reduction of nitrate to ammonia and decrease nitrite accumulation in the rumen (Takahashi *et al.* 1998, Leng 2008). However, increasing sulfur components in the diet can lead to toxic effects on the ruminants, such as reduction of sulfur to hydrogen sulfide (H_2S), which might interfere with cellular respiration or lead to formation of intermediates that complex with copper and other minerals leading to decreased mineral availability (Gould *et al.* 1997, Gould *et al.* 2002). Therefore, the sulfate reduction cannot be considered as an alternative hydrogen sink.

Bio-hydrogenation of unsaturated fatty acids

Unsaturated fatty acids can be converted into saturated fatty acids by hydrogenation, a process in which hydrogen is consumed. In 1956, Reiser & Ramakrishna Reddy reported that incubation of sheep rumen contents with linseed oil resulted in the hydrogenation of linolenic acid into linoleic acid due to microbial activity in the rumen contents (Reiser & Ramakrishna Reddy 1956). However, the rate of hydrogenation of unsaturated fatty acids depends on the type and concentration of fat delivered to the ruminants, and the ruminal pH (Van Nevel & Demeyer 1996, Beam *et al.* 2000). Fatty acids mostly constitute 3-5% of ruminant diet, mostly in the form of linolenic acid or linoleic acid, and if lipid intake was increased more than 6-9%, animals dry matter intake (DMI) decreased drastically (Palmquist & Jenkins 1980, Teh *et al.* 1994, Brown-Crowder *et al.* 2001). Therefore, there is a limit to feeding lipids, fats and oils to animals, as these can lower their ability to digest fibre, change milk composition and lower animal performance (McGinn *et al.* 2004, Zheng *et al.* 2005, Jordan *et al.* 2006). It has been shown that addition of 1% of fat results in only a 2.2% to 5.6% reduction in methane formation (Eugène *et al.* 2008, Martin *et al.* 2010).

Reductive acetogens

Reductive acetogens or homoacetogens, produce acetate from H_2 and CO_2, and are known to occur in ruminants (Leedle & Greening 1988, Henderson *et al.* 2010, Gagen *et al.* 2014). Homoacetogenesis yields billions of tons of acetate globally each year and is known to play a major role in carbon cycle (Drake 1994). However, methanogens outcompete homoacetogens at the low micromolar hydrogen concentrations present in the rumen and homoacetogens have a 100 times greater hydrogen threshold than methanogens (Breznak & Kane 1990, Mackie & Bryant 1994). Homoacetogens have hydrogen threshold concentrations in the range of 350 to 700 nM, whereas methanogens have much lower hydrogen threshold concentrations in the range of approximately 20 to 75 nM (Cord-Ruwisch *et al.* 1988). This means that methanogens can still metabolise at hydrogen concentrations at which homoacetogens cannot. Ruminal concentrations of dissolved hydrogen are often in the range of 0.2 to 2 μM (Janssen 2010), meaning that homoacetogens are often exposed to concentrations close to their thresholds, giving them limited opportunity to grow with hydrogen. Methanogens use the pathways that have more negative value of Gibb's energy change ($\Delta G°' = -134$ kJ) than homoacetogens ($\Delta G°' = -71.6$ kJ), and it is clear that species using pathways with a more negative Gibb's energy change always dominate in the environment (Cord-Ruwisch *et al.* 1988, Ungerfeld & Kohn 2006, Janssen 2010). However, homoacetogenic bacteria can also use various other substrates to produce acetate, such as carbohydrates (glucose, fructose), alcohols (ethanol, methanol), methoxylated aromatic compounds, methyl compounds, carboxylic acids, organic and halogenated compounds (Drake 1994). It is desirable to consider homoacetogenesis as alternative hydrogen utiliser in the absence of methanogenesis in the rumen, as it has two major benefits: 1) it is a hydrogen sink (four moles of H_2 are used to generate one mole of acetate) and 2) the acetate they produce captures the energy associated with the hydrogen, and represents a potential additional energy source for the animal.

There are important electron sinks present in the rumen such as propionate and butyrate production. These are intrinsic parts of the normal rumen fermentation (Wolin 1960). It is known that inhibiting methane production in the rumen results in an increase in electron flow to propionate (Janssen 2010), but incorporation of reducing equivalents not used by methanogenesis into propionate is not complete (Czerkawski 1986). Therefore hydrogen accumulates *in vitro* or *in vivo* when methane formation is inhibited

(Trei *et al.* 1971, Sauer & Teather 1987, Lee *et al.* 2009, Nollet *et al.* 1998, Mitsumori *et al.* 2012). It is therefore also important to study the contribution of propionate towards electron utilisation in the absence of methanogenesis. The other fermentation products such as butyrate and valerate also result in electron utilisation, and must also be considered.

1.3.3 Direct inhibition of methanogens using chemical compounds or vaccines

Several chemical compounds have been used in the past to reduce methane emissions from ruminants by directly inhibiting methanogens. Compounds that have been tested *in vivo* include 2-bromoethanesulfonate (BES), chloroform, bromochloromethane (BCM), 3-nitrooxypropanol (3NOP) and 9,10-anthraquinone. BES is a structural analogue of 2-mercaptoethanesulfonate (coenzyme M), involved in the last step of methane biosynthesis (Taylor & Wolfe 1974). However, inhibition of methane production by BES *in vivo* failed after 4 days, due to adaptation of methanogens to BES (Immig *et al.* 1996). Therefore, BES is not a suitable inhibitor for long-term inhibition of methane formation. BCM inhibits methanogenesis by inhibiting coenzyme B (7-mercaptoheptanoylthreoninephosphate)-dependent transferase step of methane formation (Chalupa 1977, McCrabb *et al.* 1997, Shima *et al.* 2002). BCM has been reported to reduce methane production by 30-33% and decrease the number of methanogenic archaea by 34% (Denman *et al.* 2007, Abecia *et al.* 2012). Administration of BCM complexed with α-cyclodextrin to animals resulted in a prolonged reduction in methane emissions from sheep and cattle (May *et al.* 1995, McCrabb *et al.* 1997). Methane production was reduced by approximately 91% using high doses of BCM complexed with cyclodextrin in goats without affecting feed digestibility (Mitsumori *et al.* 2012). Chloroform has also been tested *in vivo* and has been found to decrease methane emissions over 42 days in cattle (Knight *et al.* 2011). Chloroform is also known to inhibit methyl coenzyme M formation (Gunsalus & Wolfe 1978). 3-Nitrooxypropanol (3NOP) when tested in RUSITEC system and *in vivo* is a very promising methane inhibitor, without any negative effects on rumen fermentation (Haisan *et al.* 2014, Martinez-Fernandez *et al.* 2014, Romero-Perez *et al.* 2015). It resulted in a 24% reduction in methane emissions from sheep (Martinez-Fernandez *et al.* 2014) and 60% reduction in methane emissions from dairy cows (Haisan *et al.* 2014). Administration of 3NOP to lactating Holstein cows over a 12-week period decreased methane production, on average by 30%, without affecting

feed intake or milk composition (Hristov *et al.* 2015). 9,10-Anthraquinone has also been shown to reduce methane production in lambs over a period of 19 days, and it has been speculated that 9,10-anthraquinone inhibits the reduction of methyl-coenzyme M to methane by disrupting the electron transfer in methanogens (Garcia-Lopez *et al.* 1996, Kung *et al.* 1998).

Immunization of animals using vaccines that target methanogens is another strategy to directly inhibit methanogens. Immunization of animals using a vaccine that targeted less than 20% of methanogens in the rumen resulted in a 7.7% reduction in methane production (Wright *et al.* 2004). However, application of another vaccine that targeted more than 52% of different methanogen species and strains resulted in neither a decrease in methanogen numbers nor a reduction in methane emissions (Williams *et al.* 2009). Recent development of a vaccine using subcellular fractions of *Methanobrevibacter ruminantum* M1 and vaccination of sheep resulted in a decrease in methane production *in vitro* (Wedlock *et al.* 2010), but this has yet to be validated *in vivo*.

Methanogen genome analysis has been used to identify the common genes in different methanogens present in the rumen. The products of these genes could be used as methanogen-specific targets using inhibitors to mitigate ruminant methane production or a vaccine targets (Attwood & McSweeney 2008, Attwood *et al*. 2011). Gene-based discovery of inhibitors and vaccine targets is being used to develop novel control strategies for ruminant methane emissions (Leahy *et al*. 2013, Wedlock *et al*. 2013).

1.3.4 Consequences of inhibition of methane production

Chemical compounds directly toxic or inhibitory to methanogens and vaccines that inhibit methanogens over prolonged periods of time represent potentially powerful tools to mitigate methanogenesis. However, the hydrogen produced from feed fermentation, which is now not used by methanogens, could accumulate in the rumen and suppress the activity of rumen microbes, and affect fermentation (Wolin *et al.* 1997, McAllister & Newbold 2008), which may have consequences for the animal. Accumulation of hydrogen can lead to inhibition of hydrogenases that reoxidise reduced co-factors (ferredoxin, NAD(P)H and FADH). This will result in microbes using alternative pathways to recycle these reduced co-factors, and these may produce succinate, propionate, ethanol and lactate. It may result in lower acetate production and lower

adenosine triphosphate (ATP) yields, and therefore reduced microbial biomass. This could potentially slow down fibre degradation (Miller 1995, Wolin *et al.* 1997). However, if hydrogen produced which is not used by methanogens due to their inhibition, might also be lost into the environment. This is also not beneficial for the animal, as it will not be a capture of the energy in the emitted methane for the animal. It would be better if the hydrogen could be used under the anaerobic conditions of the rumen and converted into useful products such as VFA (Moss *et al.* 2000, Mitsumori & Sun 2008, Ungerfeld 2015). This trapping of hydrogen in the form of VFA would be beneficial for the animal as the source of energy.

Therefore, this problem encountered needs to be focused on for incorporation of energy associated with hydrogen for the production of fermentation products nutritionally beneficial to the ruminants. Therefore, alternative hydrogen sinks must be considered, and explored in this regard.

1.4 Overview of homoacetogens

1.4.1 Discovery and abundance of homoacetogens

Homoacetogens are strict anaerobic bacteria that are characterised by producing acetate from CO_2 as a part of their energy metabolism (Drake 1994, Muller 2003). They can grow on different substrates such as hydrogen, hexoses, formate, carbon monoxide, methoxylated compounds, and alcohols. Depending on what energy sources they are growing with, products in addition to acetate can be formed, and some can produce butyrate from CO_2 using the same basic pathway as used for acetate formation. They are known as "homoacetogens" (organisms generating only acetate), "reductive acetogens", "CO_2-reducing acetogens", or simply as "acetogens". These different terminologies are confusing, and misleading, since they do not only produce acetate, and acetogens could form acetate by any pathway. In this thesis, the term "homoacetogen" will be used, because it is a simple one word descriptor for this taxonomically-diverse group of bacteria unified by using the Wood-Ljundahl pathway to form acetate from CO_2. The term "homoacetogenesis" will be used for this metabolism, and "homobutyrogenesis" for the process in which butyrate is formed from CO_2.

In 1932, Fischer reported an organism that produced acetate using CO_2 and H_2 as described in equation (1). The first homoacetogenic bacterium was formally described in 1936 by Wieringa as *Clostridium aceticum*, but it was subsequently lost (Wieringa 1936, 1940). *Morella thermoacetica* (formerly known as *Clostridium thermoaceticum*), another homoacetogen belonging to the family *Thermoanaerobacteriaceae* was isolated from horse manure and had the unusual ability to convert glucose stoichiometrically into three moles of acetic acid (equation 2; Fontaine *et al.* 1942, Collins *et al.* 1994). Subsequently, many homoacetogens have been isolated that can metabolise hydrogen or glucose to only acetate, the origin of the name "homoacetogen".

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (1)$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (2)$$

Homoacetogens are phylogenetically diverse and 100 species of acetogens, representing 22 genera have been reported so far from different environments (Drake *et al.* 2008). Leedle and Greening discovered that reductive acetogens were abundant in the cattle rumen and isolated a novel homoacetogen species, *Acetitomaculum ruminis* (Greening & Leedle 1989). Homoacetogens have been isolated from various ruminants such as deer, bison, cattle, and sheep, and the tammar wallaby which has a digestive tract with similarities to the rumen. These homoacetogens are from wide range of taxonomic groups, and their population densities seems to be highly variable, ranging from undetectable to 10^5/ml (Le Van *et al.* 1998).

Besides acetate, homoacetogens are also known to use one-carbon compounds (CO_2 with H_2, methanol, formate, and CO) to produce butyrate, due to shift in their metabolism under certain conditions such as excess CO or H_2, or a decrease in pH (Rogers & Gottschalk 1993, Drake 1994, Drake *et al.* 2008). The formation of butyrate using CO_2 and H_2 by homoacetogens is referred to as "homobutyrogenesis" (equation (3)). In addition to the use of eight H_2 to generate two moles of acetate, and additional two moles of H_2 are used to reduce two acetate to butyrate via acetoacetate, 3-hydroxybutyrate, and crotonate, all as CoA derivatives (Kerby *et al.* 1983). Members of genera *Acetonema* and *Eubacterium* are some of the known homoacetogens that produce butyrate as well as acetate from H_2 and CO_2 (Schiel-Bengelsdorf & Dürre 2012).

$$10H_2 + 4CO_2 \rightarrow CH_3CH_2CH_2COOH + 6H_2O \quad (3)$$

1.4.2 Wood-Ljundahl pathway

Homoacetogens use the Wood-Ljungdahl pathway (Figure 1.5) to produce acetate, where an additional two moles of CO_2 are converted into one mole of acetate, using eight reducing equivalents as in equation 4 (Ljungdahl and Wood, 1969):

$$2CO_2 + 8[H] + n\text{ADP} + n\text{Pi} \rightarrow CH_3COOH + n\text{ATP} + (2+n)H_2O \quad (4)$$

Most studies of the Wood-Ljundahl pathway have been conducted with *Morella thermoacetica*. This pathway is also sometimes referred to as the reductive acetyl-CoA pathway, as it involves formation of acetyl-CoA as an intermediate in acetate formation. The Wood-Ljungdahl or reductive acetyl-CoA pathway is found in a wide range of bacteria. This pathway allows these organisms to use hydrogen as an electron donor and CO_2 as an electron acceptor, producing acetate as an end product while forming ATP (Wood 1991). The Wood-Ljundahl pathway consists of a methyl and a carbonyl branch. In the methyl branch, CO_2 is reduced to formate using hydrogen as an electron donor and tetrahydrofolate (H_4F) is attached to formate via formyltetrahydrofolate synthetase (FTHFS), resulting into formation of formyl-H_4F. During this reaction, one mole of ATP is used, and methenyl-H_4F formed. FTHFS is a key enzyme in the Wood-Ljungdahl pathway and its gene has been used to survey the diversity of homoacetogens present in different environments (Ljungdahl 1986, Lovell *et al.* 1990, Lovell & Hui 1991, Lovell & Leaphart 2005, Henderson *et al*. 2010). By using hydrogen as an electron donor, methenyl-H_4F is reduced to methylene-H_4F, and later methyl-H_4F. H_4F is separated and the methyl group transferred to a protein containing a corrinoid cofactor, and iron sulfur clusters, and this is referred to as corrinoid-iron sulfur protein (CFeSP). Next, the CFeSP is transferred to carbon monoxide dehydrogenase/acetyl-CoA synthetase (CODH/ACS) and carbon monoxide (CO) produced from a mole of CO_2 from carbonyl branch. CODH/ACS then results in the formation of acetyl-CoA, which gets phosphorylated and form acetate, releasing one mole of ATP (Schuchmann & Muller 2014). The gene for the CODH/ACS enzyme has also been used to survey the diversity of homoacetogens (McSweeney *et al.* 2009, Gagen *et al.* 2010, Matson *et al.* 2011).

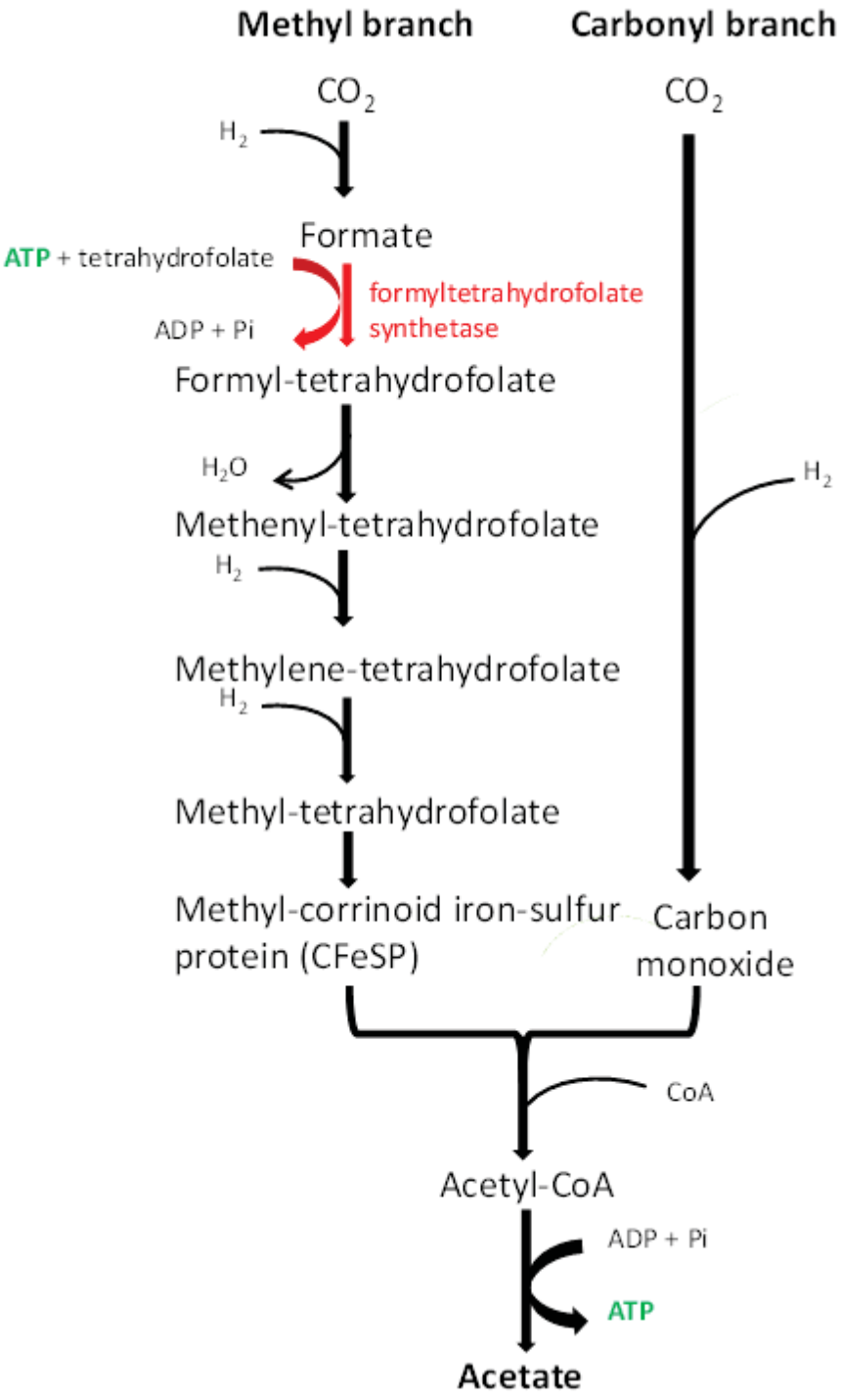

Figure 1.5 Wood-Ljungdahl pathway (Ljungdahl 1986, Wood 1991).

When growing with hydrogen (equation 1), hydrogenases catalyse the oxidation of hydrogen to protons and electrons. These electrons are the eight reducing equivalents that are required to reduce acetyl-CoA to acetate. Homoacetogens possess multiple hydrogenases with different levels of hydrogenase activity, which increases in the presence of CO_2 and H_2 (Kellum & Drake 1984, Drake 1994, Drake *et al.* 2006).

Homoacetogenic bacteria can also use diverse substrates as the source of the reducing equivalents to produce acetate (Drake 1994). One such example is formation of acetate from glucose which begins with the oxidation of glucose to pyruvate via Embden-Meyerhof-Parnas (EMP) pathway of glycolysis (Fontaine *et al.,* 1942). Pyruvate is then oxidised by pyruvate:ferredoxin oxidoreductase to acetyl-CoA, reduced ferredoxin and CO_2. Acetyl-CoA result in the formation of two moles of acetate and net generation of four moles of ATP (equation 5).

$$C_6H_{12}O_6 + 4ADP + 4Pi \rightarrow 2CH_3COOH + 2CO_2 + 4ATP + 8[H] \quad (5)$$

The eight reducing equivalents formed during EMP are then re-oxidised, and used as reducing equivalents to reduce two moles of CO_2 to form one further mole of acetate via Wood-Ljungdahl pathway (equation (4)). The net result is the fermentation of glucose to three moles of acetate (equation 2).

1.4.3 Significance of the formyltetrahydrofolate synthetase gene

Homoacetogens are highly diverse phylogenetically and versatile metabolically, and are closely related to taxa that are not homoacetogenic (Tanner & Woese 1994, Drake *et al.* 1997, Drake *et al.* 2002). Due to this polyphyletic nature of homoacetogens, 16S rRNA gene sequence analysis widely used to characterise bacteria cannot be used to identify homoacetogens. FTHFS catalyses the ATP-dependent activation of formate in the Wood-Ljungdahl pathway, and is highly conserved in both structure and function (Ljungdahl 1986, Lovell *et al.* 1990, Leaphart & Lovell 2001, Leaphart *et al.* 2003). FTHFS is encoded by the *fhs* gene, which presents a suitable alternative to 16S rRNA genes for identifying homoacetogens. A suite of *fhs* gene-directed primers is available that allows the diversity of homoacetogens and other *fhs*-containing microbes to be assessed (Leaphart & Lovell 2001, Leaphart *et al.* 2003).

Surveys of FTHFS gene sequences have been carried out in various environments, including human subgingival plaque biofilms (Vianna *et al.* 2008), the proximal and mid-colon of ostrich (Matsui *et al.* 2011), gut of giant pandas (Tun *et al.* 2014), termite gut contents (Salmassi & Leadbetter 2003, Pester & Brune 2006), tammar wallaby fore-stomach and bovine rumen (Gagen *et al.* 2010), and rumen contents from ruminants fed on different diets (Henderson *et al.* 2010). Besides the Wood-Ljundahl pathway, FTHFS

is also involved in degradation of purines and glycine as a methyltransferase in the glycine synthase-glycine reductase pathway, and in sulfate-reducing bacteria as well (Fuchs 1986, Leaphart & Lovell 2001, Lovell & Leaphart 2005). However, the FTHFS sequences from homoacetogens can be differentiated from its homologues in other pathways using both a homoacetogen FTHFS profile hidden Markov model (HoF-HMM) and homoacetogen similarity score (HS_c) based on sequences from known homoacetogens (Henderson *et al.* 2010).

1.4.4 Homoacetogens in animal gut environment

Prins and Lankhorst, were the first to suggest acetate synthesis in rats, rabbits and guinea pigs, as hydrogen was utilised in the formation of ^{14}C-acetate from $^{14}CO_2$ in ceacal contents, with no or little $^{14}CH_4$ being formed (Prins & Lankhorst 1977). These findings were later confirmed using rat faecal contents, where half of the hydrogen added was used by methanogenesis and half by homoacetogenesis (Lajoie *et al.* 1988).

Homoacetogens are known to be abundant in the guts of lower and higher termite (Breznak & Switzer 1986, Breznak 1994), wood-eating cockroaches (Cleveland *et al.* 1935, Breznak & Switzer 1986) and human colon (Lajoie *et al.* 1988). In the termite gut, homoacetogenesis is the dominant hydrogen sink and acetate formed serves as the major energy source for termites (Odelson & Breznak 1983, Pester & Brune 2007). In the human intestinal tract, homoacetogens decreased the intestinal hydrogen concentration and resulted in increased acetate formation in colonic content (Kamlage *et al.* 1997). In the tammar wallaby and kangaroos, gut fermentation results in less methane formation than in ruminants (Kempton *et al.* 1976, Von Engelhardt *et al.* 1978, Dellow *et al.* 1988), which might be attributed to more efficient homoacetogens present in these animals relative to ruminants (Gagen *et al.* 2010). Homoacetogenesis also serves as the major pathway for hydrogen removal in the cecum and proximal colon of ostrich, as compared to mid colon where methanogenesis dominates (Fievez *et al.* 2001). Besides animal guts and human colon, homoacetogens have also been reported to play a major role as hydrogen-scavengers in microbial electrochemical cells (Parameswaran *et al.* 2012), and out-competed methanogens for hydrogen during batch-start up in a membrane biofilm reactor (Ziv-El *et al.* 2012).

Homoacetogens form part of the microbiota in the rumens (or rumen-like fore stomachs) of newly born lambs (Morvan *et al.* 1994, Doré *et al.* 1995), beef and dairy cattle (Leedle & Greening 1988, Greening & Leedle 1989, Jiang *et al.* 1995, Joblin 1999), deer (Rieu-Lesme *et al.* 1995), llamas and bison (Rieu-Lesme *et al.* 1996). Although homoacetogenesis seems feasible in these systems, there are various reasons attributed to methanogens outcompeting homoacetogens in ruminants described earlier. The available evidence suggests that significant homoacetogenesis cannot occur in the rumen unless interventations are taken to increase their competitiveness. There have been several studies carried out to determine the role of homoacetogenesis in the rumen and promote homoacetogenesis, which are described in the following section.

1.4.5 Significance of homoacetogenesis in ruminants

Inhibition of methane formation and utilisation of excess hydrogen by homoacetogenesis might lead to an increase in microbial growth yield and benefit animal productivity (Joblin 1999). Homoacetogens can out-compete methanogens only when hydrogen availability is not limiting, or when there are excess of homoacetogens in the rumen. This can be achieved by either suppressing the growth of methanogens and allowing natural resident homoacetogens to use the hydrogen available or by artifically increasing the hydrogen concentration *in vitro* (Immig *et al*. 1996, Le Van *et al.* 1998, Nollet *et al.* 1998). Whether homoacetogens can act as an alternative hydrogen sink in the rumen has been investigated *in vitro* and *in vivo* (Table 1.1).

Table 1.1 *In vitro* and *in vivo* studies of rumen contents to determine the role of homoacetogens.

	Additions	Effect on CH_4 production	Effect on H_2 concentration	Homoacetogenesis	Persistence of the effect	References
Addition of homoacetogens *in vitro*						
Co-culture experiment involving acetogen *Blautia* sp. Ser8, methanogen *Methanobrevibacter* sp. MF_2 and live yeast cells *Saccharomyces cerevisiae* as feed additive, grown under elevated H_2 pressure (80%)	Ser8 + MF_2 Ser8 + yeast cells Ser8 + MF_2 + yeast cells	↓ by 14.2% Zero ↓ by 27.3%	NR NR NR	↑ in acetate production was observed. Although labelled carbon incorporation into acetate was not determined, homoacetogenesis was likely.	5 days incubation.	Chaucheyras *et al.* (1995)
Short-term *in vitro* incubation of cow rumen contents with BES and/or addition of acetogens (A10 and ^3H isolates)	^3H + BES A10 + BES	↓ by 99.6% ↓ by 98.8%	↑ by 4-fold ↑ by 40-fold	NR NR	74 h	Boccazzi & Patterson (1996)
Addition of *Blautia producta* (*Peptostreptococcus productus*) U1 during short-term incubation of rumen contents or addition of BES	*B. producta* (100% CO_2) *B. producta* + 0.03 mM BES (100% CO_2) *B. producta* (80% H_2 + 20% CO_2) *B. producta* + 0.03 mM BES (80% H_2 + 20% CO_2)	100% ↓ by 94.9% 100% ↓ by 95.8%	↑ by 1-fold ↑ by 95-fold ↑ by 1-fold ↑ by 100-fold	NR NR NR NR	24 h	Nollet *et al.* (1997)

	Additions	Effect on CH_4 production	Effect on H_2 concentration	Homoacetogenesis	Persistence of the effect	References
Rumen contents incubated with the cell-free supernatant of *Lactobacillus plantarum* 80 and *Blautia producta*	*Lactobacillus plantarum* 80 (1.5 ml) *Lactobacillus plantarum* 80 (1.5 ml) + *B. producta* (5 ml)	↓ by 10.5% ↓ by 15.3%	↑ by 100-fold ↑ by 1-fold	NR NR	Persisted for only 24 h. During long-term *in vitro* (72 h) incubation and *in vivo* (24 days) addition, these effects did not last for long	Nollet *et al.* (1998)
Addition of rumen acetogen, and BES *in vitro*	5 mM BES +100% H_2 + $NaH^{13}CO_3$ + *Acetitomaculum ruminis* 190A4	Not detected	Detected	↑ in acetogenic activity by 2-fold	45 h	Le Van *et al.* (1998)
Six different acetogens added into the rumen fluid incubated *in vitro* in the presence and absence of BES	*Eubacterium limosum* strain Eggerth or *Blautia* sp. Ser5 *Eubacterium limosum* strain Eggerth + BES *Blautia* sp. Ser5 + BES	↓ by 5%	Not detected ↓ by 4-fold ↓ by 1-fold	NR NR NR	24 h	Lopez *et al.* (1999)
Existence of homoacetogenesis in rumen						
Existence of hydrogenotrophic bacteria in lambs	$^{14}CO_2$ and $^{13}CO_2$ incorporation	Not formed	NR	Double labelled acetate was formed	24 h old lamb	Morvan *et al.* (1994)

	Additions	Effect on CH_4 production	Effect on H_2 concentration	Homoacetogenesis	Persistence of the effect	References
Inhibition of methanogenesis to enhance homoacetogenesis						
Infusion of BES into sheep rumen	BES	Recovered after 4 days of infusion	No accumulation after 4 days of infusion	failed	4 days	Immig *et al.* (1996)

↑, Increase; ↓, decrease; NR, not reported.

1.4.6 Measure homoacetogenesis and VFA inter-conversion in rumen

In the past, acetate formation from CO_2 has been investigated by incorporation of $^{14}CO_2$ into acetate in caecal contents of rodents and in bovine rumen fluid (Prins & Lankhorst 1977), termite guts (Breznak & Switzer 1986, Tholen & Brune 1999), and in peat soil samples (Ye *et al.* 2014). During *in vitro* incubation of bovine rumen contents, acetate formation was measured by addition of $NaH^{13}CO_3$ and 100% H_2 (v/v) in the presence or absence of the homoacetogen *Acetitomaculum ruminis* 1904A and the methanogen inhibitor BES (Le Van *et al.* 1998). Morvan et al. (1994) also demonstrated the incorporation of $NaH^{13}CO_3$ into acetate in the presence of 100% H_2 (v/v) during incubation of washed cell preparations from new born lambs. However, ^{13}C-label is also known to appear in propionate besides acetate during incubation of bovine rumen contents and kangaroo fore-stomach contents with $NaH^{13}CO_3$ (Godwin *et al.* 2014). Therefore, it is important to account for acetate that might come from propionate or butyrate due to VFA inter-conversion. Moreover, in rumen studies, VFA concentrations are often used to describe VFA production, but the concentration-production relationship may be compromised by VFA inter-conversions (Nolan *et al.* 2014). VFA inter-conversion refers to appearance of label into one or more VFA during infusion of labelled VFAs (Leng & Brett 1966, Glinsky *et al.* 1976, Sharp *et al.* 1982, Bruce *et al.* 1987, Sutton *et al.* 2003, Ungerfeld & Kohn 2006, Nolan *et al.* 2014). Several studies have revealed that there is extensive VFA inter-conversion between acetate and butyrate in both *in vitro* and *in vivo* studies. An *in vitro* study by Esdale and co-workers showed that addition of lactate increased the conversion of acetate to butyrate and decreased the reverse reaction, i.e., conversion of butyrate to acetate (Esdale *et al.* 1968). Another study on VFA production rates in the cecum of the pony using a mixture of 1-^{14}C-acetate, 1-^{14}C-propionate and 2,3-^{3}H-butyrate revealed that half of the butyrate is derived from acetate and only 2-4% of acetate comes from butyrate due to VFA inter-conversion (Glinsky *et al.* 1976). A study on sheep rumen fluid reported that considerable inter-conversion occurred between acetate and butyrate. Some 51-66% of the carbon in butyrate was derived from acetate and 15-28% of the carbon in acetate came from butyrate. However, inter-conversions between propionate and acetate or butyrate were comparably small (Bergman *et al.* 1965, Esdale *et al.* 1968). Infusion of 2-^{13}C-propionate into sheep suggested that 1.8% of acetate came from propionate and 8.1% of butyrate from

propionate (Van Der Walt & Briel 1976). A three-compartment model to describe the kinetics of VFA inter-conversion during infusion of ^{14}C-acetate, ^{14}C-propionate and ^{14}C-butyrate into sheep for 240 min also showed that the major inter-conversion was between butyrate and acetate rather than propionate to acetate or butyrate (Nolan *et al.* 2014).

These inter-conversions mean that any measures of label incorporation into products, especially small amounts, need to take into account the inter-conversions. Since homoacetogenic activity is expected to be low in the rumen, care has to be taken to confirm that homoacetogenesis is real and not due to some artefact of VFA inter-conversion.

1.5 Rationale of the study

Ruminant livestock support the sustenance of millions of people in the world, but contribute significantly to global GHGs. Over 32% of New Zealand's GHGs can be attributed to methane formed during microbial breakdown of feed in the digestive tract of ruminants such as sheep and cattle (Ministry for the Environment, 2014). Micro-organisms called methanogens form methane from hydrogen produced during feed fermentation in the rumen, and lead to a loss of feed energy for the animal and environmental consequences. Reducing these emissions will have an immediate benefit for the environment and may also have a positive impact on the animal's energetic efficiency. Proposed methane mitigation strategies such as dietary manipulation, feed additives, inhibitors, and vaccines have been developed or are being developed to reduce methane emissions from ruminants (Section 1.3). However, inhibiting methane production may cause accumulation of unused hydrogen in the rumen, which may decrease the rate of feed conversion, i.e., slow down rumen fermentation and thereby affect animal productivity. One of the greatest challenges is to re-direct the hydrogen to some other useful end-products, or understand the flow of the electrons represented by that hydrogen, in the absence of methanogens.

Homoacetogens are potentially beneficial hydrogen-utilisers and these have been the major focus of the studies presented in this thesis. Homoacetogens are a group of microbes known to reside in the rumen, and which can use H_2 and CO_2 to

form acetate, an important substrate for ruminant metabolism. However, homoacetogens are unable to compete with the methanogens and reach a level of the dominance, because they have a higher threshold for hydrogen than methanogens. When methanogens are inhibited, for example using inhibitors or vaccines, homoacetogens may play a role in using hydrogen generated in the rumen fermentation. Therefore, the overall aim was to inhibit the methanogens using chemical inhibitors, and to study if homoacetogenesis becomes a significant hydrogen (or electron) utilising process. In the future, knowledge of these hydrogen utilising micro-organisms can be used in conjunction with methane mitigation strategies to facilitate the transition from a normal methane-producing rumen to an equally or even more productive low methane one.

1.6 Research questions and outline of the thesis

The work presented in this thesis will allow an assessment to be made of the potential of homoacetogensis to take over the role of methanogensis. The original aim of the research described in this thesis was to identify alternative hydrogen utilisers in the rumen, with an emphasis on homoacetogens. The first step was to ensure that homoacetogens were actually active in the rumen. Methods to measure homoacetogenesis were therefore developed, and then experiments performed to verify that what was measured was true homoacetogenesis and not an artefact of chemical reactions occurring in the rumen fluid. After developing these methods, homoacetogenesis was measured in sheep rumen fluid (*in vitro* and *in vivo*) in the absence and presence of methanogenesis. Ultimately, this work showed that homoacetogens were active, but the inital aim of identifying those in the rumen was not addressed. Thus, the thesis will mainly report on the development of protocols to estimate homoacetogenic activity that can be used to investigate the impacts of future methane mitigation strategies. The various research questions are:

1) What happens to ruminal fermentation and hydrogen utilisation when methanogenesis is slowed or halted using methanogen-specific inhibitors?
2) Can homoacetogenesis be measured in sheep rumen contents by following $^{13}CO_2$ incorporation into acetate, and differentiated this activity from other processes?

3) Does homoacetogenesis increase during inhibition of methanogenesis in *in vitro* serial batch fermentations or in the sheep rumen, and can the hydrogen that is not used by methanogens be incorporated into acetate?
4) What is the significance of homoacetogenesis as a hydrogen or electron sink in the presence and absence of methanogenesis?
5) What alternative pathways of ruminal hydrogen or electron utilisation become active when methanogens are inhibited?

An outline of the thesis chapters with their objectives is given below in Figure 1.6.

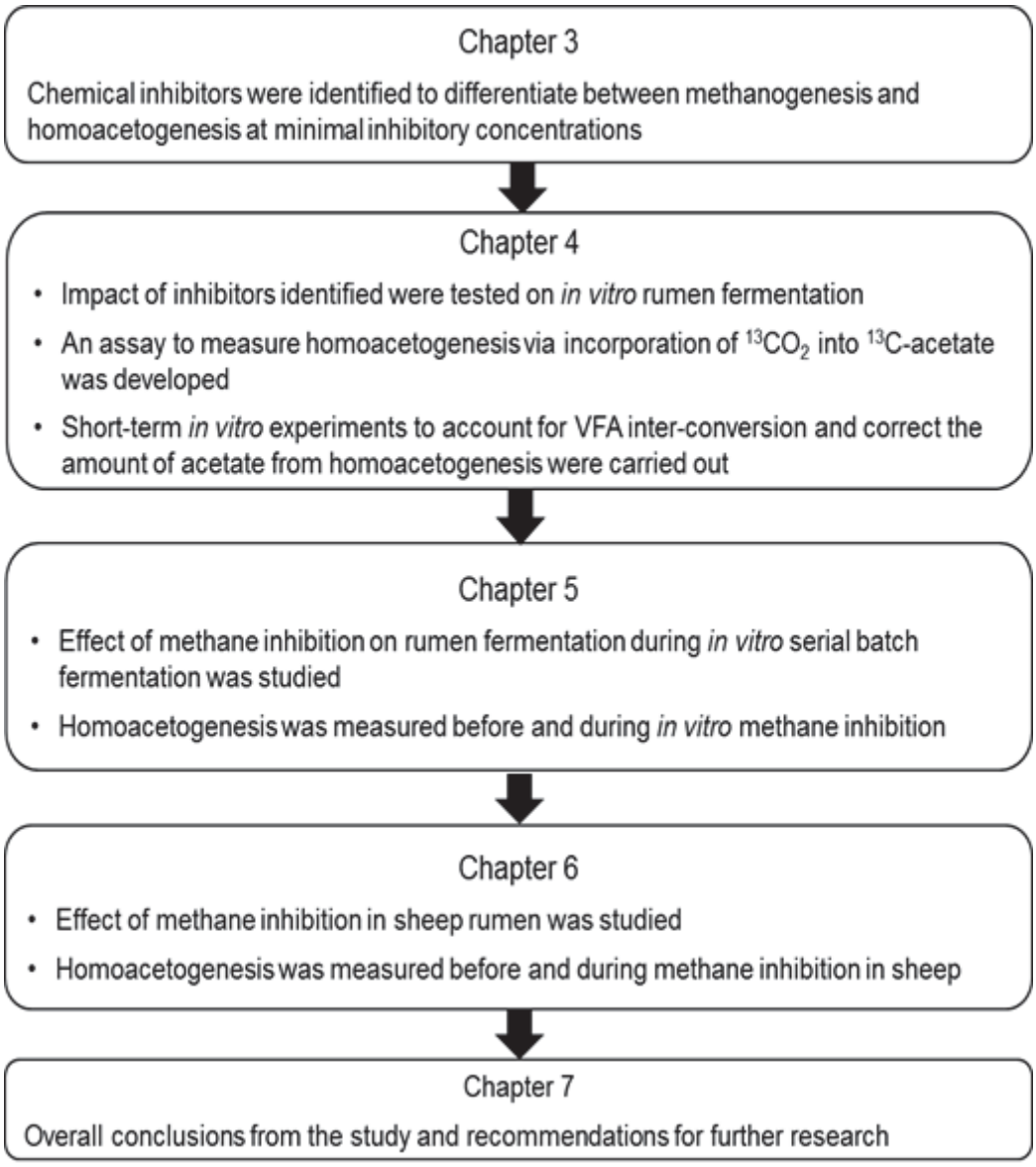

Figure 1.6 Outline of the thesis.

Chapter 2

General methodology

2.1 Animal use

Collection of rumen contents for medium preparation from cows or for mixed culture experiments from sheep was approved by AgResearch Grasslands Animal Ethics Committee. Maintenance of fistulated sheep on general purpose (GP) diet was approved by Animal Ethics Committee under approval AE#12391. All aspects of the sheep trial carried out in Chapter 6 were approved by the Grasslands Animal Ethics Committee approval AE#12908.

2.2 Collection of rumen fluid for preparation of medium and other additives

Rumen contents were collected from fistulated cows that were fed meadow hay. Feed was withheld from the cows in the evening (4 p.m.) and the rumen fluid was collected next morning (8 to 9 a.m.). The rumen contents were filtered through a cheese cloth with the mesh size of approximately 1 mm (Stockinette Cirtex Industries Ltd., Thames, New Zealand). The fine particulate material was removed by centrifugation at 10,000 g for 20 min (Sorvall Evolution RC, Thermo Fisher Scientific Inc., MA, USA). The supernatant (rumen fluid) was then frozen at –20 ºC.

2.3 Rumen fluid and medium additives

The processing of rumen fluid and preparation of various additives added to the medium used for growth of microorganisms (methanogens, homoacetogens or other bacteria) are described below:

2.3.1 Base clarified rumen fluid

The rumen fluid stock (Section 2.2) was thawed at room temperature and centrifuged at approximately 20,000 g at 4 °C for 15 min (Sigma 3-18K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was bubbled with nitrogen (N_2) for 10 min in serum bottles (120 ml; BellCo Glass,

Vineland, NJ, USA). The serum bottles were sealed with black butyl rubber seals (BellCo Glass, Vineland, NJ, USA) and aluminium caps (BellCo Glass, Vineland, NJ, USA) and autoclaved at 121 ºC for 15 min (to inactivate viruses). Then, it was allowed to cool and 1.63 g $MgCl_2.6H_2O$ and 1.18 g $CaCl_2.2H_2O$ were added per 100 ml of rumen fluid. A heavy precipitate was formed, which was removed by centrifugation at 30,000 *g* at 4 ºC for 60 min. The supernatant was referred to as the "clarified rumen fluid" (Kenters *et al.* 2011).

2.3.2 No Substrate Rumen Fluid Vitamin mix (NoSubRFV)

Two grams of yeast extract were added per 100 ml of clarified rumen fluid. This was mixed and then bubbled with N_2 gas for 15 min and transferred to a sealed and sterile N_2-flushed serum bottle through a 0.22 µm pore size Millex GP sterile syringe filter (Millipore Corp., Bedford, MA, USA) using a sterile syringe and needle. Two millilitres of Vitamin 10 concentrate (Section 2.3.3) were added per 100 ml of this preparation using a sterile syringe and needle (Kenters *et al.* 2011). For use, 0.5 ml of NoSubRFV was added to 9 ml medium.

2.3.3 Vitamin 10 concentrate

The following components were added per litre of distilled water: 0.04 g 4-aminobenzoate, 0.010 g d-(+)-biotin, 0.1 g nicotinic acid, 0.05 g hemicalcium D-(+)-pantothenate, 0.15 g pyridoxamine hydrochloride, 0.1 g thiamine chloride hydrochloride, 0.05 g cyanocobalamin, 0.03 g D,L-6,8-thioctic acid, 0.03 g riboflavin and 0.01 g folic acid (Janssen *et al.* 1997, Kenters *et al.* 2011). The solution was bubbled with N_2 for 15 min and then transferred into sealed and sterile N_2-filled (100%) serum bottles through a sterile 0.22 µm pore size Millex GP sterile syringe filters using a sterile syringe and needle. The bottles were wrapped in aluminium foil to protect the vitamins against light and stored at 4 °C or frozen at –20 °C.

2.3.4 Selenite/tungstate solution

The following components were dissolved per litre of distilled water: 0.5 g NaOH, 0.003 g $Na_2SeO_3.5H_2O$ and 0.004 g $Na_2WO_4.2H_2O$. This was dispensed in 100 ml aliquots of the solution in Schott bottles and autoclaved at 121 °C for 20 min (Tschech & Pfennig 1984).

2.3.5 Trace element solution SL10

The following components were dissolved per litre of distilled water: 10 ml HCl (25%), 1.5 g $FeCl_2.4H_2O$, 0.19 g $CoCl_2.6H_2O$, 0.1 g $MnCl_2.4H_2O$, 0.07 g $ZnCl_2$, 0.006 g H_3BO_3, 0.036 g $Na_2MoO_4.2H_2O$, 0.024 g $NiCl_2.6H_2O$ and 0.002 g $CuCl_2.2H_2O$ (Widdel *et al.* 1983). This was dispensed in 100 ml aliquots of the solution in Schott bottles and autoclaved at 121 °C for 20 min.

2.3.6 Salt solution A

Salt solution A was prepared by dissolving the following components per litre of distilled water: 6 g NaCl, 3 g KH_2PO_4, 1.5 g $(NH_4)_2SO_4$, 0.79 g $CaCl_2.2H_2O$ and 1.2 g $MgSO_4.7H_2O$.

2.3.7 Salt solution 2B

Salt solution 2B was prepared by dissolving 7.86 g $K_2HPO_4.3H_2O$ per litre of distilled water.

2.3.8 Acetate (1 M):methanol (1 M):formate (3 M)

The following components were dissolved in 50 ml distilled water: 6.80 g sodium acetate, 2.03 ml methanol and 10.20 g sodium formate. The volume was made up to 100 ml by adding more distilled water and then bubbled with N_2 for 30 min. The solution was transferred to a sealed and sterile N_2-flushed serum bottle through a 0.22 μm pore size Millex GP sterile syringe filter using a sterile syringe and needle. For use, 0.2 ml of this solution was added to 10 ml medium to achieve final concentrations of 20 mM acetate, 20 mM methanol, and 60 mM formate.

2.3.9 Glucose (1 M)

Eighteen grams of D-glucose was dissolved in 100 ml of distilled water. The solution was bubbled with N_2 for 30 min and then transferred into a sealed and sterile N_2-flushed serum bottle through a 0.22 μm pore size Millex GP sterile syringe filter using a sterile syringe and needle. For use, 0.05 ml of this solution was added to 10 ml medium to achieve a final concentration of 5 mM glucose.

2.3.10 GCXAL-CPY-Rumen Fluid-Vitamin mix (2GenRFV)

The following components were dissolved per 100 ml of clarified rumen fluid: 0.72 g D-glucose, 0.68 g D-cellobiose, 0.60 g D-xylose, 0.60 g L-arabinose, 1.76 ml Na L-lactate syrup (50%), 4 g casamino acids (4% w/v), 4 g Bacto-Peptone (4% w/v)

and 4 g yeast extract (4% w/v). This was mixed and bubbled with N_2 gas for 15 min, then transferred to a sealed and sterile N_2-flushed serum bottle through a 0.22 µm pore size Millex GP sterile syringe filter using a sterile syringe and needle. Two millilitres of Vitamin 10 concentrate (Section 2.3.3) was added per 100 ml of this preparation using a sterile syringe and needle, and the mix was stored at 4 °C.

2.3.11 Pectin (10% w/v)

Five grams of pectin was added to 50 ml distilled water and stirred, then this was transferred to a serum bottle and bubbled with CO_2 for 30 min. The serum bottle was then sealed with a butyl rubber stopper and aluminium cap and autoclaved at 121 °C for 15 min. The solution was kept at 4 °C. For use, 1 ml of 10% pectin was added to 9 ml medium to achieve a final concentration of 1% (w/v)

2.3.12 Co-enzyme M (100 mM)

This was prepared by dissolving 0.82 g sodium 2-mercaptoethanesulfonate (co-enzyme M; Sigma-Aldrich, Saint Louis, MO, USA) in 50 ml distilled water, adding it to a serum bottle and bubbling with N_2 for 30 min. The solution was transferred into a sealed and sterile N_2-flushed serum bottle through a 0.22 µm pore size Millex GP sterile syringe filter using a sterile syringe and needle. The bottle was wrapped with aluminium foil to protect the co-enzyme M from light, and kept at 4 °C. For use, the 100 mM stock solution was further diluted to 1 mM in distilled water in a N_2-flushed Hungate tube, and 0.1 ml of this 1 mM solution was added to 9 ml medium to achieve a final concentration of 10 µM.

2.4 BY medium

BY medium (Joblin *et al.* 1990) consisted of following components per litre: 170 ml salt solution A (Section 2.3.6), 170 ml salt solution 2B (Section 2.3.7), 300 ml base clarified rumen fluid (Section 2.3.1), 360 ml distilled water, 1 ml selenite/tungstate solution (Section 2.3.4), 1 ml trace element solution SL10 (Section 2.3.5), 10 drops of resazurin solution (0.1% w/v) and 1 g yeast extract. The components were thoroughly mixed and boiled for few minutes in a microwave oven. The medium was cooled in an ice bath while it was continuously bubbled with 100% CO_2. Once cooled, 0.5 g L-cysteine.HCl and 5 g $NaHCO_3$ were added and mixed gently. The medium was dispensed in 9-ml aliquots into CO_2-filled

Hungate tubes (16 mm diameter, 125 mm long; BellCo Glass, Vineland, NJ, USA) and sealed using black butyl rubber seals and plastic caps. The tubes were autoclaved at 121 °C for 20 min at 15 psi and warmed to 39 °C in a darkened room before use.

2.5 RM02 medium

RM02 medium was prepared by dissolving following components per litre: 1.4 g K_2HPO_4, 0.6 g $(NH_4)_2SO_4$, 1.5 g KCl, 1 ml selenite/tungstate solution (Section 2.3.4), 1 ml trace element solution SL10 (Section 2.3.5) and 4 drops of resazurin solution (0.1% w/v) in 950 ml of distilled water (Kenters *et al.* 2011). The components were thoroughly mixed and boiled for few minutes in a microwave oven. The medium was cooled in an ice bath while it was continuously bubbled with 100% CO_2. Once cooled, 4.2 g $NaHCO_3$ and 0.5g L-cysteine.HCl were added per litre. The medium was dispensed in 9-ml aliquots into CO_2-filled Hungate tubes and sealed using black butyl rubber seals and plastic caps. The tubes were autoclaved at 121 °C for 20 min at 15 psi and warmed to 39 °C in a darkened room before use.

2.6 Pure culture experiments

Media, additives and substrates required for the growth of methanogens, homoacetogens and other bacteria used in this study are described in Table 2.1. All the cultures were grown anaerobically using the Hungate technique (Bryant 1972, Macy *et al.* 1972).

Table 2.1 Methanogen, homoacetogen and bacterial cultures used, and the media, substrates, and additives required for their growth.

	Genus/Species	Medium	Substrates and additives
Methanogens			
AbM4	*Methanobrevibacter* sp.	RM02	NoSubRFV, 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate, $H_2 + CO_2$[a]
BRM9	*Methanobacterium formicicum*	RM02	NoSubRFV, 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate, $H_2 + CO_2$[a]
SM9	*Methanobrevibacter millerae*	RM02	NoSubRFV, 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate, $H_2 + CO_2$[a]
M1	*Methanobrevibacter ruminantium*	RM02	NoSubRFV, 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate, $H_2 + CO_2$[a]
ISO3-F5	*Methanosphaera* sp.	RM02	NoSubRFV, 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate, $H_2 + CO_2$[a]
YLM1	*Methanobrevibacter* sp.	RM02	NoSubRFV, 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate
ISO4-H5	Methanomasiliicoccales Group 12 sp.	BY	Vitamin mix, CoM (10 µM), 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate, filtered culture of *Succinovibrio dextrinosolvens* H5 (grown in BY medium and 1% pectin (w/v))
D5	*Methanobrevibacter* sp.	BY	Vitamin mix, CoM (10 µM), 20 mM sodium acetate, 20 mM methanol, 60 mM sodium formate
Homoacetogens			
Ser5	*Blautia* sp.	RM02	NoSubRFV, 5 mM glucose or , $H_2 + CO_2$[a]
Ser8	*Blautia* sp.	RM02	NoSubRFV, 5 mM glucose or , $H_2 + CO_2$[a]
SA11	*Eubacterium limosum*	RM02	NoSubRFV, 5 mM glucose or , $H_2 + CO_2$[a]
Other bacteria			
B316	*Butyrivibrio proteoclasticus*	RM02	2GenRFV
23	*Prevotella ruminicola*	RM02	2GenRFV
FD1	*Ruminococcus flavefaciens*	RM02	2GenRFV

	Genus/Species	Medium	Substrates and additives
GA-192	*Selenomonas ruminantium*	RM02	2GenRFV
S85	*Fibrobacter succinogenes*	RM02	2GenRFV
Ga6A1	*Bacteroides* sp.	RM02	2GenRFV

[a]Pressurised with H_2+CO_2 to 1 atm over pressure after adding all other components.

2.7 Culture growth measurement techniques

The growth of methanogens, homoacetogens or other bacteria was followed using the following techniques:

2.7.1 Phase contrast microscopy

Preparations were made by adding a small amount of culture to a microscopy slide using a sterile syringe and needle. The slides were viewed under phase contrast and UV-fluorescence optics using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany) using a 100× oil phase contrast objective.

2.7.2 Gas chromatography

The growth of methanogens was monitored by measuring methane production using an Aerograph 660 (Varian Associats, Palo Alto, CA, USA) fitted with a Porapak Q 80/100 mesh column (Waters Corporation, Milford, MA, USA) and a thermal conductivity detector operated at 100 °C. Headspace gas, 0.3 ml, from methanogen culture tube was injected into the GC using a sterile syringe and needle fitted with a luer-lock valve. N_2 gas was used as a carrier gas at 12 ml/min at room temperature.

2.7.3 Spectrophotometer

The growth of methanogens, homoacetogens and other bacteria was followed by measuring changes in the optical density in a spectrophotometer (Spectronic 200, Thermo Fischer Scientific, Mumbai, India). The culture density was measured at 600 nm by inserting the culture tubes (Hungate tubes) into the spectrophotometer.

2.8 Preservation of cultures in DMSO and regeneration of frozen cultures (methanogens, homoacetogens and other bacteria)

Dimethyl sulfoxide (DMSO; 50% v/v) was prepared by mixing the following components to make up 100 ml of solution: 17 ml salt solution A (Section 2.3.6) ,

17 ml salt solution 2B (Section 2.3.7), 16 ml DMSO, 50 ml distilled water, 0.5 g $NaHCO_3$ and 2 drops of resazurin solution (0.1% w/v). The components were thoroughly mixed and boiled for a few minutes in a microwave oven. The solution was cooled in an ice bath while it was continuously bubbled with 100% CO_2. Once cooled 0.05 g L-cysteine.HCl was added and the solution mixed gently. The solution was dispensed in 10-ml aliquots into CO_2-filled Hungate tubes and were sealed using black butyl rubber seals and plastic caps. The tubes were autoclaved at 121 °C for 20 min at 15 psi and stored in the dark, as DMSO is light sensitive. One millilitre of this anaerobic and sterile DMSO solution was injected into culture tubes containing a grown culture to achieve the final concentration of 5% (v/v) DMSO. Methanogen cultures were also pressurized with H_2/CO_2 (80:20 v/v) to 1atm overpressure after adding DMSO. The cultures were kept at –20 °C for 1-2 h and then transferred to –85 °C freezer.

Cultures frozen at –85 °C using DMSO were thawed at room temperature or in a beaker containing warm water. Once thawed, 0.5 ml or 1 ml of the culture was inoculated into fresh medium (BY or RM02) in Hungate tubes and using appropriate substrates and additives. The tubes were pressurized with H_2/CO_2 (80:20 v/v) for methanogens and incubated at 39 °C in the dark on a shaker (200 rpm, Infors HT Labotron, Bottmingen, Switzerland).

2.9 Preparation of inhibitors for pure culture studies

The preparation of inhibitors used for screening against pure cultures of methanogens, homoacetogens or other bacteria is described below.

2.9.1 Sodium 2-bromoethanesulfonate (BES)

BES was prepared in distilled water. The highest stock concentration of 500 mM BES was prepared by dissolving 2.64 g sodium 2-bromoethanesulfonate (Sigma-Aldrich, Saint Louis, MO, USA) in 25 ml of distilled water. The solution was gassed with N_2 for 30 min, and then filtered through a sterile 0.22 μm pore size Millex GP sterile syringe filters using a sterile syringe and needle. The other stock concentrations were prepared as described in Table 2.2. For use, 0.2 ml of a suitable stock solution was added to 10 ml of growth medium in Hungate tubes.

Table 2.2 Concentrations of BES tested, stock solutions prepared and amounts added to Hungate tubes.

Conc. tested (µM)	Stock solution (µM)	Amount added (ml)
2	100	0.2
5	250	0.2
10	500	0.2
20	1000	0.2
50	2500	0.2
1000	50000	0.2
2000	100000	0.2
5000	250000	0.2
10000	500000	0.2

2.9.2 Chloroform

Chloroform ($CHCl_3$) was prepared in RM02 medium. RM02 medium was prepared as described above (Section 2.5) under CO_2 in serum bottles. The highest stock concentration of 250 mM chloroform was prepared by dissolving 504 µl chloroform (AnalaR, BDH, England) in 25 ml RM02 medium. The chloroform was added drop-by-drop into 25 ml RM02 medium in a serum bottle using a sterile syringe and needle, and mixed gently. The other stock concentrations prepared are described in Table 2.3. For use, 0.2 ml of a suitable stock solution was added to 10 ml of growth medium in Hungate tubes.

2.9.3 Acetylene

Acetylene gas provided in commercial and purified grade cylinder is dissolved in acetone and known to contain various contaminants such as H_2, ammonia (NH_3), H_2S, CH_4, arsine (ArH_3) and phosphine (PH_3). In order to purify acetylene, the gas provided in the cylinder (BOC NZ, Auckland, NZ), was bubbled into concentrated sulfuric acid (conc. H_2SO_4) as described by (Hyman & Arp 1987). Sulfuric acid is most efficient in removing acetone and phosphine and traces of other impurities. After scrubbing with conc. H_2SO_4, the gas was bubbled into 5 M sodium hydroxide (NaOH) to remove sulfur trioxide (SO_3) and H_2S, and collected and stored under water in an immersed glass tube capped at one end with a rubber septum to facilitate gas removal via gas tight syringes fitted with luer lock taps and needles. The desired stock concentrations (Table 2.4) were then prepared by adding the acetylene gas to

Table 2.3 Concentrations of $CHCl_3$ tested, stock solutions prepared and amounts added to Hungate tubes.

Conc. tested (µM)	Stock solution (µM)	Amount added (ml)
2	100	0.2
5	250	0.2
10	500	0.2
20	1000	0.2
50	2500	0.2
100	5000	0.2
200	10000	0.2
500	25000	0.2
1000	50000	0.2
2000	100000	0.2
5000	250000	0.2

Table 2.4 Concentrations of acetylene tested, stock solutions prepared and amounts added to Hungate tubes.

Conc. tested (µM)	Stock solution (% v/v)	Amount added (ml)
2	0.2	0.5
5	0.5	0.5
10	1	0.5
20	2	0.5
50	5	0.5
100	10	0.5
1000	100	0.5
2000	200	0.5

previously sealed, capped and autoclaved serum bottles containing seven glass beads (2 mm), to facilitate gas mixing upon shaking and N_2 gas. The desired dilutions were prepared by diluting the acetylene gas in the serum bottles with N_2 gas, which was also added using a sterile syringe and needle. The bottles were shaken well in order to mix the two gases properly and 0.5 ml of this dilution was added to Hungate tubes containing 10 ml of growth medium. The amounts added were calculated to ensure that the dissolved acetylene concentrations were achieved after equilibration of the gas between the gas and liquid phases. The amount of acetylene to be added to acheive a target concentration of dissolved gas was calculated according to Henry's law, i.e., $A_d = P/K_T$, where A_d is the concentration

of the dissolved acetylene, P is the partial pressure of the gas in the headspace and K_T is the temperature-corrected Henry's law constant, taking into account that the set-up and incubations were done at different temperatures (T). Henry's law constant, $K°$, for acetylene was 0.041 mol/l × atm (at 25 °C), the temperature correction constant for acetylene was 1800, and the incubation temperature used for cultures was 38 °C, and the set-up temperature when handling gases was taken as 25 °C. K_T was calculated from $K°$ and C, as $K_T = K°e^{(C\,(1/T - 1/298.15)}$.

2.9.4 Fluoroacetate

To prepare a 250 mM stock solution of fluoroacetate, 0.25 g sodium monofluoroacetate was dissolved in 10 ml distilled water. The solution was gassed with N_2 for 30 min and then filtered through a sterile 0.22 µm pore size Millex GP sterile syringe filters using a sterile syringe and needle. The other stock concentrations prepared are described in Table 2.5. For use, 0.2 ml of a suitable stock solution was added to 10 ml of growth medium in Hungate tubes.

2.9.5 *n*-butyl isocyanide

A solution of 500 mM *n*-butyl isocyanide (Thermo Fisher Scientific New Zealand Ltd., Auckland, New Zealand) was prepared by dissolving 0.27 ml *n*-butyl isocyanide in 5 ml distilled water, and a 100 mM stock was prepared by dissolving 0.05 ml *n*-butyl isocyanide in 5 ml distilled water. The two solutions were continuously gassed with N_2 for 30. The solution was then filtered through through a sterile 0.22 µm pore size Millex GP sterile syringe filters using a sterile syringe and needle. For use, 0.1 ml of a suitable stock solution was added to Hungate tube containing 10 ml of growth medium (Table 2.6).

2.9.6 Phosphate

A 2 M stock of phosphate was prepared by dissolving 13.6 g KH_2PO_4 and 22.8 g $K_2HPO_4.3H_2O$ in 25 ml distilled water. A 1 M stock was prepared by dissolving 6.8 g KH_2PO_4 and 11.4 g $K_2HPO_4.3H_2O$ in 25 ml distilled water. The pH was 6.3 and the final volume was made up to 50 ml for each stock solution. The two solutions were gassed with N_2 for 30 min and then filtered through a sterile 0.22 µm pore size Millex GP sterile syringe filters using a sterile syringe and needle. The amounts added to Hungate tubes growth medium are provided in Table 2.7.

Table 2.5 Concentrations of fluoroacetate tested, stock solutions prepared and amounts added to Hungate tubes.

Conc. tested (µM)	Stock solution (µM)	Amount added (ml)
10	500	0.2
20	1000	0.2
100	5000	0.2
200	10000	0.2
1000	50000	0.2
2000	100000	0.2
3000	150000	0.2
4000	200000	0.2
5000	250000	0.2

Table 2.6 Concentrations of *n*-butyl isocyanide tested, stock solutions prepared and amounts added into the Hungate tubes.

Conc. tested (µM)	Stock solution (mM)	Amount added (ml)
1000	100	0.1
5000	500	0.1

Table 2.7 Concentrations of phosphate tested, stock solutions prepared and amounts added to Hungate tubes.

Conc. tested (µM)	Stock solution (M)	Amount added (ml)
10000	1	0.1
20000	1	0.2
50000	2	0.25
100000	2	0.5

2.9.7 1,10-phenanthroline

1,10-phenanthroline was prepared by dissolving 0.45 g 1,10-phenanthroline in 5 ml water to obtain a 500 mM stock, and 0.09 g 1,10-phenanthroline in 5 ml water to obtain a 100 mM stock. The two stock solutions were gassed with N_2 for 30-40 min and then filtered through a sterile 0.22 µm pore size Millex GP sterile syringe filters using a sterile syringe and needle. For use, 0.1 ml of a suitable stock solution was

added to 10 ml of growth medium in Hungate tubes, to obtain the desired concentration (Table 2.8).

Table 2.8 Concentrations of 1,10-phenanthroline tested, stock solutions prepared and amounts added into the Hungate tube.

Conc. tested (μM)	Stock solution (mM)	Amount added (ml)
0	0	0.1
1000	100	0.1
50000	500	0.1

2.9.8 Rifampicin

Rifampicin (Sigma-Aldrich, Saint Louis, MO, USA) stock solutions were prepared in 5 ml of distilled water (Table 2.9). The stock solutions were gassed with N_2 for 30 min and then filtered through a sterile 0.22 μm pore size Millex GP sterile syringe filters using a sterile syringe and needle. For use, 0.1 ml of the stock solutions was added to 10 ml of growth medium in Hungate tubes, to obtain the desired concentration (Table 2.9).

Table 2.9 Concentrations of rifampicin tested, stock solutions prepared and amounts added into the Hungate tube.

Conc. tested (mg/ml)	Stock solution (mg/ml)	Amount added (ml)
0.005	0.5	0.1
0.01	1	0.1
0.05	5	0.1

2.9.9 5, 5'-dithio-bis-(2-nitrobenzoic acid; also known as Ellman's reagent)

Ellman's reagent was prepared by dissolving 0.495 g Ellman's reagent (Thermo Fisher Scientific New Zealand Ltd., Palmerston North, New Zealand) to 5 ml DMSO (100%) to obtain a 250 mM stock concentration. The headspace of this was gassed with N_2 for 30 min and the solution then filtered through a sterile 0.22 μm pore size Millex GP sterile syringe filters using a sterile syringe and needle. For use, 0.2 ml of a suitable stock solution was added to 10 ml of growth medium in Hungate tubes, to obtain the desired concentration (Table 2.10). Control cultures contained the same volume of DMSO, prepared in the same way.

Table 2.10 Concentrations of Ellman's reagent tested, stock solutions prepared and amounts added into the Hungate tube.

Conc. tested (µM)	Stock solution (µM)	Amount added (ml)
1	50	0.2
100	5000	0.2
1000	50000	0.2
5000	250000	0.2

2.10 DNA extraction from pure cultures

DNA was extracted from pure cultures of methanogens and homoacetogens. The cultures were grown in BY or RM02 medium and 1.8 ml of the cultures were pelleted by centrifugation at 14,000 *g* for 1 min in sterile microfuge tubes using a Minispin personal microcentrifuge (Eppendorf, Hamburg, Germany). The supernatant was removed and 200 µl of InstaGene matrix (Bio-Rad Laboratories Inc., Hercules, CA, USA) was added to the pellet. This was incubated at 56 °C for 15-30 min and then vortexed at high speed for 10 s (Labnet, Woodbridge, NJ, USA). The tubes were placed in a 100 °C heat block for 8-10 min and vortexed again at high speed for 10 s. The cell debris was removed by centrifugation at 12,000 *g* for 5 min. The resulting supernatant contained the DNA and was stored at –20 °C.

2.11 Agarose gel electrophoresis

1% (w/v) agarose gel was prepared in 1× TAE buffer. The solution was boiled to dissolve and then, SYBR safe DNA gel stain (Invitrogen, Carlsbad CA, USA) at a concentration of 2× was added. PCR products were loaded into wells with 20% (v/v) Orange G loading buffer (Sigma-Aldrich, St Louis, USA). A standard 1kb$^+$ DNA ladder (Invitrogen, Carlsbad CA, USA) was loaded into the wells of the first and last lane. The gel was run in 1× TAE buffer in either a Wide mini-Sub Cell GT electrophoresis system (Bio-Rad Laboratories Inc., California, USA) or an Owl A2 Large Gel System (Thermo Fisher Scientific Inc., MA, USA) at 60-100 V for 45-60 min. The bands were then visualized under UV trans-illumination and image was taken using a Gel Logic 200 imaging system (Eastman Kodak, New York, NY, USA).

50× TAE buffer was prepared by mixing following components per litre of distilled water: 242 g Tris, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA. The pH was adjusted to 8.0 and final volume was made up by adding distilled water. 1× TAE was prepared by diluting 50× TAE by 50-fold using distilled water and adjusted the pH to 8.0.

2.12 DNA quantification

DNA was quantified using a Quant-iT dsDNA Broad range (BR) or Quant-iT dsDNA High sensitivity (HS) assay kit (Invitrogen, Oregon, USA) by either of the two methods according to instructions provided by the manufacturer using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) or a Qubit fluorometer (Invitrogen, Carlsbad, USA).

2.13 Polymerase chain reaction (PCR)

All the PCR cycles were run on Mastercycler pro S (Eppendorf AG, Hamburg, Germany). Primers used were ordered from IDT (Custom Science, Auckland, New Zealand) or Invitrogen (Invitrogen, Carlsbad, USA). The different conditions, reagents, and primers used during PCRs are described in Table 2.11.

2.14 PCR product purification

PCR products were purified using the following kit according to the manufacturer's instructions using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA).

2.15 Extraction and purification of DNA from agarose gels

DNA fragments were excised from agarose gels using a sterile and sharp scalpel under UV trans-illumination. DNA was extracted from the gel slice and purified according to the manufacturer's instructions using the QIAquick gel extraction kit (Qiagen, Venlo, The Netherlands).

Table 2.11 Primers, PCR conditions and components used to amplify specific genes.

Primer pairs	Primer sequence (5'-3')	Target gene	Fragment size	PCR reaction (50 µl)	PCR conditions	Reference
27f 1492r	GAG TTT GAT CMT GGC TCA G GGY TAC CTT GTT ACG ACT T	16S rRNA (bacteria)	~1465 bp	Taq buffer (+ 15 mM Mg^{2+}) 5 µl, Mg^{2+} (25 mM) 2 µl, Taq (5 U/µl) 0.2 µl, dNTP (2 mM) 5 µl, water 33.8 µl, primers (5 pmol/l) 1 µl each and DNA 2 µl	94 °C for 4 min 94 °C for 1 min 55 °C for 1 min 72 °C for 1 min 72 °C for 10 min	(Lane 1991, Sait *et al*. 2002)
					25 cycles	
915af 1386r	AGG AAT TGG CGG GGG AGC AC GCG GTG TGT GCA AGG AGC	16S rRNA (archaea)	~471 bp	Taq buffer (+ 15 mM Mg^{2+}) 5 µl, Taq (5 U/µl) 0.5 µl, dNTP (2 mM) 4 µl, water 38.5 µl, primers (100 pmol/l) 0.5 µl each and DNA 1 µl	95 °C for 2 min 95 °C for 15 s 59 °C for 30 s 72 °C for 1 min 72 °C for 7 min	(Skillman *et al*. 2004, Watanabe *et al*. 2004)
					30 cycles	
FTHFSf FTHFSr	TTY ACW GGH GAY TTC CAT GC GTA TTG DGT YTT RGC CAT ACA	FTHFS	1100 bp	Taq buffer (+ 15 mM Mg^{2+}) 5 µl, Taq (5 U/µl) 0.4 µl, dNTP (2 mM) 5 µl, water 27.4 µl, primers (5 pmol/l) 5 µl each, BSA (10%) 0.2 µl and DNA 2 µl	94 °C for 2 min 94 °C for 30 s 53 °C for 30 s 72 °C for 1 min 72 °C for 10 min	(Leaphart & Lovell 2001, Henderson *et al*. 2010)
					30 cycles	

Primer pairs	Primer sequence (5'-3')	Target gene	Fragment size	PCR reaction (50 µl)	PCR conditions	Reference
GEM2945f site GEM189r site	CTG CAA GGC GAT TAA GTT GGG AGC GGA TAA CAA TTT CAC ACA GG	FTHFS cloning	~1465 bp	Taq PCR master mix (Taq buffer: 1.5 mM $MgCl_2$ + Taq (2.5 U/µl) + dNTP (0.2 mM)) 25 µl, Mg^{2+} (25 mM) 1 µl water 23 µl, primers (10 pmol/l) 0.5 µl each and small amount of colony	94 °C for 1 min 94 °C for 15 s 56 °C for 30 s 72 °C for 1 min (35 cycles) 72 °C for 7 min	(Sait *et al.* 2002, Taylor *et al.* 2002)
GEM2987f TOP168r	CCC AGT CAC GAC GTT GTA AAA CG ATG TTG TGT GGA ATT GTG AGC GG	TOPO cloning		Taq buffer (+ 15 mM Mg^{2+}) 5 µl, Mg^{2+} (25 mM) 1 µl, Taq (5 U/µl) 0.5 µl, dNTP (2 mM) 10 µl, water 32.5 µl, primers (10 pmol/l) 0.5 µl each and small amount of colony	94 °C for 2 min 94 °C for 15 s 56 °C for 30 s 72 °C for 1 min (30 cycles) 72 °C for 7 min	(O'Farrell & Janssen 1999, Osborne *et al.* 2005)

M represents equimolar A and C; Y represents equimolar C and T; W represents equimolar A and T; D represents equimolar G, T and A; R represents equimolar A and G; H represents equimolar C, T and A.

2.16 Cloning for qPCR plasmid preparation

Amplified PCR products (archaea using primers 915af and 1386f and bacteria using primers 27f and 1492r) were ligated into pCR 2.1 TOPO TA cloning vector (Invitrogen) and transformed into TOP10 chemically-competent *Escherichia coli* cells (One Shot TOP10 cells; Invitrogen) according to the protocol and instructions provided by the manufacturer (Invitrogen). After transformation, the cells were plated on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 40 mg/ml), ampicillin (50 mg/ml) and isopropyl β-D-1-thiogalactopyranoside (IPTG; 100 mM) and incubated overnight at 37 °C. White colonies were selected randomly and were streaked on LB agar plates containing ampicillin (50 mg/ml) and incubated overnight. Some of these streaked colonies were amplified by colony PCR protocol using GEM2987f (10 pmol/µl) and TOP168r (10 pmol/µl) primers and checked on agarose gel and subjected to Sanger sequencing at the Allan Wilson Centre Genome Sequencing Service (Massey University, Palmerston North, New Zealand). The sequencing results were obtained as ABI files and were analysed and edited using the ContigExpress package of Vector NTI (VectorNTI Advance 11, Invitrogen, Carlsbad, USA). The streaked colonies which gave positive sequencing results were grown in LB medium containing ampicillin (50 mg/ml). Plasmids were isolated using the QIA plasmid isolation kit (Qiagen) and quantified using the Quant-iT dsDNA Broad range (BR) assay kit (Invitrogen) in a Qubit fluorometer.

2.16.1 LB (Luria-Bertani) medium

The following components were mixed per litre of distilled water: 10 g Bacto-tryptone, 5 g yeast extract and10 g NaCl. The pH was adjusted to 7.0 and the final volume was made up by adding distilled water. The medium was transferred to screw capped Hungate tubes and autoclaved at 121 °C for 15 min.

2.16.2 LB agar plates with ampicillin

These were prepared by adding 15 g of agar per litre of LB medium. This was autoclaved at 121 °C for 15 min, allowed to cool to 50 °C, before ampicillin was added (50 mg/ml). Aliquots of 30-35 ml of this medium were poured into sterile disposable 85 mm Petri dishes and left until the agar had set. These plates were stored at 4 °C.

2.16.3 LB agar plates with ampicillin/IPTG/X-Gal

LB ampicillin plates were prepared (Section 2.16.2) and then 100 µl IPTG (100 mM) and 20 µl X-Gal (40 mg/ml) were spread over the surface of each LB ampicillin plate and allowed to dry for 30 min at 37 °C before use.

2.16.4 Preservation of clones in glycerol and regeneration of frozen clones (archaea and bacteria)

Clones of *Escherichia coli* grown overnight at 37 °C in LB medium containing ampicillin (50 mg/ml) were preserved for future use by adding sterile glycerol (30% v/v). The cultures were kept in a −85 °C freezer.

Clones of *Escherichia coli* frozen at −85 °C were regenerated by streaking a small aliquot of the frozen material on LB agar plates containing ampicillin (50 mg/ml) and incubated overnight at 37 °C.

2.17 Assessment of the microbial community composition

The microbial community (archaea and bacteria) were analysed using following techniques.

2.17.1 454 pyrosequencing

DNA was extracted using the PCQI method (Rius *et al.* 2012, Henderson *et al.* 2013) which employs phenol-chloroform with bead beating and the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands). To do this, 0.7 g of zirconium beads (0.1 mm diameter) were added to a screw-capped bead beating vial and autoclaved at 121 °C for 15 min. Then, 30 mg freeze-dried rumen contents were added to the autoclaved vials containing the beads. 200 µl of 20% (w/v) sodium dodecyl sulfate, 282 µl Buffer A (100 mM NaCl, 200 mM, 200 mM Tris, 20 mM EDTA, pH 8), 268 µl of Buffer PB (from the QIAquick PCR purification kit) and 550 µl of phenol/chloroform/isoamylalcohol (25:24:1) at pH 8 were added to the vials. The vials were shaken for 4 min at full speed in a Mini-Beadbeater-96 (Biospec Products, Bartlesville, OK, USA) and then placed on ice. The samples were centrifuged for 20 min at 16,000 *g* and 4 °C, and then placed on ice. 500 µl of the supernatant was collected and transferred into 1.5 ml microfuge tubes. 650 µl of Buffer PB was added and then extraction followed the manufacturer's

instructions for the QIAquick PCR Purification Kit. The extracted DNA was quantified using Quant-iT dsDNA Broad range (BR) assay kit in a Qubit fluorometer. The extracted DNA was normalised to a 40 ng/µl concentration using a Eppendorf Epmotion robot (Eppendorf AG, Hamburg, Germany).

Bacterial and archaeal 16S rRNA gene regions were amplified in triplicate as described previously (Rius *et al.* 2012). All primers (Integrated DNA Technologies Inc., Coralville, IA, USA) consisted of 454 Titanium adapter sequences A (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3') or B (5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG-3'), a two-base linker sequence between the barcode and the group-specific primer, and a unique 12-base error-correcting Golay barcode attached to adapter A for sample identification followed by the specific primer sequence. The specific primers used for archaea were Ar915af (5'-AGG AAT TGG CGG GGG AGC AC-3') and Ar1386Rr (5'-GCG GTG TGT GCA AGG AGC-3'), and for bacteria were Ba27f (5'-GAG TTT GAT CMT GGC TCA G-3') and Ba515Rmod (5'-CCG CGG CKG CTG GCA C-3'). Amplicons from the two microbial groups were quantified fluorometrically using the Quant-iT dsDNA Broad range (BR) assay kit in a Qubit fluorometer, normalised per sample, and pooled per microbial group. A total of 1 µg DNA from each of the two resulting pools was loaded onto an agarose gel (1%, w/v). Bands were visualized and excised under blue light trans-illumination, and amplicons were gel-purified with the QIAquick Gel Extraction Kit (Qiagen). The amplicons were quantified using the Quant-iT dsDNA High sensititivity (HS) assay kit in a Qubit fluorometer and diluted to obtain 1×10^9 copies/µl for the two microbial groups. The two pools were mixed together to obtain a ratio of 5:1 (bacteria: archaea). Bacterial and archaeal amplicons were sequenced using 454 GS FLX Titanium chemistry at Eurofins MWG Operon (Ebersberg, Germany).

2.17.2 qPCR (Quantitative real time-PCR)

To estimate microbial numbers, marker loci for bacteria and archaea were enumerated in freeze-dried rumen samples by quantitative PCR (Jeyanathan *et al.* 2011) using a SYBR Green I fluorescence kit (LightCycler 480 SYBR Green I Master or LightCycler FastStart DNA Master SYBR Green I kits, Roche Diagnostics GmbH, Mannheim, Germany) on a Rotor-Gene 6000 real-time rotary analyser (Corbett Life Science, Concorde, NSW, Australia).

2.17.2.1 qPCR for archaea

Absolute archaeal 16S rRNA gene numbers were quantified using the LightCycler 480 SYBER Green I Master Kit (Roche Diagnostics GmbH, Mannheim, Germany). The standards for archaea were prepared using plasmids containing 16S rRNA genes amplified from *Methanobrevibacter ruminantium* strain M1 (DSM 1093), *Methanomicrobium mobile* strain BP (1539) and *Methanosphaera* ISO3-F5. The plasmids were quantified using Quant-iT dsDNA Broad range (BR) kit in a Qubit fluorometer. The three plasmids were then mixed together in equimolar concentration. The plasmid mix was diluted 10-fold each time and a series of standards from 10^{-2} to 10^{-10} were prepared. The plasmid standards were run in duplicate. The reactions were set up in a Gene-Disc 100 (Corbett Life Science, Concorde, NSW, Australia). A 20 µl reaction was set-up for each sample and each plasmid standard. Each 20 µl reaction contained 1 µl primer 915af (1 µM), 1 µl primer 1386r (1 µM), 10 µl LC480 LightCycler 480 Master Mix, 6 µl nuclease-free water and 2 µl DNA sample. Each reaction was carried out using two different dilutions of DNA (1:10, 1:50 for *in vitro* and 1:50, 1:100 for *in vivo* samples) and in triplicate for each dilution. The disc was sealed with adhesive film (Corbett Life Science, Concorde, NSW, Australia) and the quantification was run on a Rotor-Gene 6000 real-time rotary analyser. The PCR cycling conditions involved initial denaturation at 95 °C for 10 min, followed by 40 cycles for amplification (denaturation at 95 °C for 10 sec, annealing at 59 °C for 5 sec, extension at 72 °C for 10 sec). After each run, melting curves were evaluated between 72 °C and 95 °C to check for non-specific signals and high quality target amplification. The results were analysed using Rotorgene 6000 series software version 1.7 (Corbett Life Science) and archaeal 16S rRNA copy numbers were calculated for each dilution and yield of DNA determined in per ml of liquid fermentation sample (*in vitro* trial; Chapter 5) or per g of rumen content (*in vivo* trial; Chapter 6).

2.17.2.2 qPCR for bacteria

Absolute bacterial 16S rRNA gene numbers were quantified using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics GmbH, Mannheim, Germany). The standards for bacteria were prepared using three plasmids (BAC15, BAC18 and BAC23; Jeyamalar, 2010) containing 16S rRNA gene fragments amplified using the bacteria-specific primers 27f and 1492r. The plasmids were quantified using Quant-iT dsDNA Broad range (BR) kit in a Qubit fluorometer. The

three plasmids were then mixed together in equimolar concentration. The plasmid mix was diluted 10-fold each time and a series of standards from 10^{-2} to 10^{-10} were prepared. The plasmid standards were run in duplicate. The reactions were set up in a Gene-Disc 100 (Corbett Life Science). A 20 µl reaction was set-up for each sample and plasmid standards used. Each 20 µl reaction contained 2 µl primer 519f (1 µM), 2 µl primer 907r (1 µM), 2.4 µl $MgCl_2$ (4 mM), 2 µl Light Cycler Mix, 0.4 µl bovine serum albumin (1% BSA; Invitrogen), 9.2 µl nuclease-free water and 2 µl DNA sample. Each reaction was carried out using two different dilutions of DNA (1:50, 1:100 for both *in vitro* and *in vivo* samples) and in triplicates for each dilution. The disc was sealed with adhesive film (Corbett Life Science) and was run on a Rotor-Gene 6000 real-time rotary analyser. The PCR cycling conditions involved initial denaturation at 95 °C for 10 min, followed by 40 cycles for amplification (denaturation at 95 °C for 10 sec, annealing at 52 °C for 5 sec, extension at 72 °C for 10 sec). After each run, melting curves were evaluated between 72 °C and 95 °C to check for non-specific signals and high quality target amplification. The results were analysed using Rotorgene 6000 series software version 1.7 (Corbett Life Science) and bacterial 16S rRNA copy numbers were calculated for each dilution and yield of DNA determined in per ml of liquid fermentation sample (*in vitro* trial; Chapter 5) or per g rumen content (*in vivo* trial; Chapter 6).

2.17.3 Phylogenetic analysis of microbial pyrosequencing reads

Pyrosequencing data were processed and analysed using the QIIME (Quantitative Insights Into Microbial Ecology) software package version 1.5 (Caporaso *et al.* 2010). Sequences over 400 bp in length with an average quality score over 25 were assigned to a specific sample via 12-base error-correcting Golay barcodes. Each read was assigned to a sample using the barcode information and quality control was performed. The split_libraries.py command along with -l 400 (minimum sequence length), -b golay_12 (barcode type), -r (remove unassigned reads), -n 1 (sequence i.d.) and -z truncate_only (removes the primer and subsequent sequence data from the output read, and does not alter output of sequences where the primer cannot be found) was used as quality control. On average, there were 1280 archaeal and 7521 bacterial reads per sample in the *in vitro* trial (Chapter 5; Appendix 2; Table A.2.2) and 1445 archaeal and 6546 bacterial reads per sample in the *in vivo* trial (Chapter 6; Appendix 3; Table A.3.4) available for analysis after quality control. The sequence reads were not denoised, as denoising can result in

substantial reductions in the diversity at the OTU and phylogenetic levels (Reeder & Knight 2010). The results from denoised and non-denoised data are highly correlated to each other (Reeder & Knight 2010). It has also been shown that non-denoising had no marked effects on the rumen methanogenic community (Seedorf *et al*. 2015). The samples were also not normalised or rarefied before analysis. Operational taxonomic units (OTUs) were generated by clustering reads sharing >97% sequence similarity and taxonomic assignments were done on these OTUs. The uclust method was used for clustering reads with 97% similarity, and the cut-off E-value for OTU assignment to taxa was 1e-10. Sequences were assigned to phylogenetic groups by BLAST (Altschul *et al.* 1990) of bacterial 16S rRNA genes against the Greengenes database (version gg_13_5; McDonald *et al.* 2012), and of archaeal 16S rRNA genes against RIM-DB database (Seedorf *et al.* 2014) independently. Relative abundance values were calculated for each taxon. Bacterial data were summarized at phylum, class, order, family and genus levels, but analyses used the data at the genus level. Where taxa were not be defined below the family or order level, those groups were used in the analysis as if they were genera. Archaeal data were summarized using a mixed taxonomic rank scheme (Janssen & Kirs 2008).

2.18 Mixed culture experiments

The medium used for mixed culture experiments, the inhibitors and ^{13}C-labelled tracers added, along with the procedures used for sample collection, processing and analysis are provided in the sections below.

2.18.1 Mould's medium

Mould's medium is defined as the buffer used to dilute rumen contents five-fold for *in vitro* (mixed culture) incubations. Mould's medium comprises of the combination of bicarbonate and phosphate salts (Mould *et al.* 2005). It was prepared as follows:

- 10× Buffer 1 (Macrominerals): The following components were dissolved in 5 litre of distilled water: 42.57 g Na_2HPO_4 (60 mM), 65.10g KH_2PO_4 (95.7 mM) and 5.25 g $MgCl_2 \cdot 6H_2O$ (5.2 mM).
- 10× Buffer 2: The following components were dissolved in 5 litre of distilled water: 270.90 g $NaHCO_3$ (644.9 mM) and 70.35 g NH_4HCO_3 (178.1 mM).

To make up a final volume of 3 litres of buffered (Mould's medium) rumen contents, 240 ml 10× Buffer 1, 240 ml 10× Buffer 2, 1920 ml distilled water and 600 ml filtered rumen fluid were mixed. This was kept in a water bath at 39 °C with continuous CO_2 being bubbled into it for 1 h. Part way through that step, 0.94 g L-cysteine.HCl and 0.6 ml NaOH (10 N) were added after 40-45 min.

2.18.2 Preparation of inhibitors for *in vitro* studies

The inhibitors BES, chloroform and acetylene were prepared using the same methods as described in Section 2.9.1, 2.9.2 and 2.9.3 respectively. The only difference in the preparation of inhibitors was that they were not prepared anaerobically for *in vitro* experiments as compared for pure culture studies.

2.18.3 Preparation of unlabelled VFA for *in vitro* studies

The unlabelled VFA (acetate, propionate and butyrate) were prepared according to Table 2.12.

Table 2.12 Preparation of unlabelled VFA for addition *in vitro*.

Stock solution (mM)	Amounts added per 10 ml of distilled water (g)	Working solution (mM)	Amounts added to 60 ml medium (ml)
Sodium acetate trihydrate			
900	1.22	15	1
1800	2.44	30	1
2700	3.67	45	1
3600	4.89	60	1
Sodium propionate			
900	0.86	15	1
1800	1.72	30	1
Sodium butyrate			
600	0.66	10	1
1200	1.32	20	1

2.18.4 Preparation of ^{13}C-labelled tracers

- ^{13}C-sodium bicarbonate solution ($NaH^{13}CO_3$; Sigma-Aldrich, Saint Louis, MO, USA): A 5% enriched $NaH^{13}CO_3$ solution was prepared by dissolving 84 mg $NaH^{13}CO_3$ in 5 ml distilled water in a falcon tube (Corning Inc., New York, NY, USA). One millilitre of this solution was added to 60 ml rumen fluid diluted

five-fold (v/v) in Mould's medium in serum bottles prior *in vitro* incubation, to achieve a final concentration of 3 mM.

- $^{13}C_2$-sodium acetate solution (Sigma-Aldrich, Saint Louis, MO, USA): A 1.8 M stock solution of $^{13}C_2$-sodium acetate was prepared by dissolving 1.5 g $^{13}C_2$-sodium acetate in 10 ml distilled water in a Schott bottle. 1 ml of this stock solution was added to 60 ml rumen fluid diluted five-fold with Mould's medium (v/v) in serum bottles immediately prior *in vitro* incubation to achieve a final concentration of 30 mM.
- 2-$^{13}C_1$-sodium propionate solution (Sigma-Aldrich, Saint Louis, MO, USA): A 0.9 M stock solution of 2-$^{13}C_1$-sodium propionate was prepared by dissolving 0.87 g 2-$^{13}C_1$-sodium propionate in 10 ml distilled water in a Schott bottle. One millilitre of this stock solution was added to 60 ml rumen fluid diluted five-fold with Mould's medium (v/v) in serum bottles immediately prior *in vitro* incubation to achieve a final concentration of 15 mM.
- $^{13}C_3$-propionic acid solution (Sigma-Aldrich, Saint Louis, MO, USA): A 0.9 M stock solution of $^{13}C_3$-propionic acid was prepared by dissolving 0.7 ml $^{13}C_3$-propionic acid in 9.3 ml distilled water in a Schott bottle. One millilitre of this stock solution was added to 60 ml rumen fluid diluted five-fold with Mould's medium (v/v) contained in serum bottles immediately *in vitro* incubation to achieve a final concentration of 15 mM. One millilitre of sodium hydroxide (NaOH; 0.9 M) was also added to the serum bottle to neutralise the $^{13}C_3$-propionic acid.
- $^{13}C_4$-sodium butyrate solution (Sigma-Aldrich, Saint Louis, MO, USA): A 0.6 M stock solution of $^{13}C_4$-sodium butyrate was prepared by dissolving 0.68 g $^{13}C_4$-sodium butyrate in 10 ml distilled water in a Schott bottle. One millilitre of this stock solution was added to 60 ml rumen fluid diluted five-fold with Mould's medium (v/v) in serum bottles immediately *in vitro* incubation to achieve final concentration of 10 mM.

2.18.5 Sample collection from *in vitro* bottles and processing

- Liquid fermentation samples (for VFA analysis): 1.8 ml liquid fermentation samples (*in vitro* trials) or rumen fluid samples (*in vivo* trial) were collected in 2 ml microfuge tubes. The samples were centrifuged at 21,000 *g* (Thermo Scientific Heraeus Fresco 21 centrifuge, Thermo Electron LED GmbH,

Osterode am Harz, Germany) for 10 min at 4 °C. Of the resulting supernatant, 900 µl was mixed with 100 µl internal standard (19.7 mM 2-ethylbutyric acid in 20% (v/v) orthophosphoric acid; Sigma-Aldrich, Saint Louis, MO, USA) and the mixture was stored at −85 °C for future analysis. The rest of the supernatant was discarded, and the pellet was stored at −20 °C for DNA analysis (*in vitro* trials). At the time of analysis, the VFA samples were thawed at room temperature and centrifuged again at 21,000 *g* for 10 min at 4 °C. 800 µl of the supernatant was added to 1.5 ml crimp top GC vials. Samples were analysed using a Hewlett Packard HP6890 series gas chromatograph system (Hewlett-Packard, Wilmington, DE, USA) fitted with a HP-FFAP column (length 30 m; internal diameter 0.53 mm; film thickness 1.0 µm; J&W Scientific, Folsom, CA, USA) equipped with a deactivated fused silica guard column. One microlitre of sample was injected on the column with a HP6890 series injector (Hewlett-Packard, Wilmington, DE, USA). Helium was used as the carrier gas at a flow rate of 5 ml/min. The oven temperature was set at 85 °C at the start of the run for each sample, ramped to 200 °C at 10 °C/min, held at 200 °C for 10 min and then reduced to 50 °C, and held for 5 min before the next sample was injected. Acetate, propionate and butyrate were measured using this method. The method has previously been described by Attwood *et al.* (2006).

- Liquid fermentation samples (for branched and short-chain fatty acids analysis): Branched and short-chain fatty acids such as isobutyrate, isovalerate, valerate, caproate, lactate, formate and ethanol were measured using HPLC (Ehrlich *et al.* 1981). For this, 1.8 ml of liquid fermentation samples (*in vitro*) or rumen fluid samples (*in vivo* trial) were collected in 2 ml microfuge tubes. The samples were centrifuged at 21,000 *g* for 10 min at 4 °C. The resulting supernatant was stored at −85 °C before HPLC analysis. At the time of analysis, the samples were thawed at room temperature and centrifuged again at 21,000 *g* for 10 min at 4 °C. One millilitre of the supernatant was filtered through a 0.22 µm pore size PVDF syringe filters (Thermo Fisher Scientific New Zealand Ltd., Auckland, New Zealand) into 2 ml vials with a cap (Phenomenex NZ Ltd., Auckland, New Zealand) . The samples were then run on a Shimadzu LC10vp HPLC (Shimadzu Oceania Ltd., Auckland, New Zealand) fitted with a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm) and a Shimadzu RID 10A refractive index detector. The temperature was maintained at 45 °C and the

injection volume of sample was 50 µl. The mobile phase was 5 mM sulfuric acid in water, and the flow rate was 0.8 ml/min.

- Headspace gas samples (to determine the $^{13}C/^{12}C$ ratio in CO_2 and CH_4 using GC-C-IRMS): One millilitre of headspace gas samples were collected from each bottle using a sterile syringe and needle. The sample was then added to a 6-ml exetainer (soda glass vials; Labco Ltd., Lampeter, Ceredigion, UK) which were previously evacuated and filled with N_2 gas. The samples were analysed by isotope ratio mass spectrometry (IRMS) using a Thermo Delta V+ (Thermo Scientific, ThermoFisher Scientific Inc, Waltham, MA, USA) attached to a Conflow IV continuous flow interface and a Thermo Trace gas chromatography (GC) equipped with Conflow III micro Pt/Ni/Cu oxidation and Cu reduction furnaces and nafion drier and a GC Pal autosampler. The samples (25 µl) were injected onto a CP-PoraPLOT Q GC capillary column (length 25 m; internal diameter 0.32 mm; film thickness 10 µm; Varian, Crawford Scientific, Scotland, UK) using a 1:20 split ratio and helium as the carrier gas. The column was maintained isothermally at 65 ^oC. The eluted gas components were passed through the oxidation and reduction furnaces operating at 940 ^oC and 650 ^oC respectively, converting the CH_4 to CO_2 prior to entering IRMS. The IRMS monitored ions at m/z 44, 45 and 46 corresponding to the isobaric species of CO_2. The ion chromatograms were integrated and the peak $^{13}C/^{12}C$ ratio for CH_4 and CO_2 was calculated relative to a calibrated CO_2 reference gas analysed at start of every sample analysis and corrected for ^{17}O via SSH algorithm (Santrock *et al.* 1985).
- Dissolved liquid fermentation samples (to determine the $^{13}C/^{12}C$ ratio in CO_2 using GC-IRMS): 150 µl liquid fermentation samples that had been centrifuged were collected and added to 6-ml exetainers that had previously been evacuated and contained 50 µl of orthophosphoric acid and N_2 gas. The acidification drove dissolved gasses into the headspace of the exetainers. The samples were stored at 4 °C and analysed by GC- IRMS as described above.
- Liquid fermentation samples (to determine the $^{13}C/^{12}C$ ratio in acetate, propionate, and butyrate using solid phase micro extraction (SPME) and GC-IRMS): One millilitre liquid fermentation samples were collected from each bottle in 6-ml exetainers. These were stored at −85 °C. At the time of processing, the samples were thawed and 100 µl orthophosphoric acid was added and mixed properly. The vials were kept at 45-50 °C for 1 min in a

heating block and then SPME fibre (75 µm carboxen/polydimethysiloxane; Supelco, Sigma-Aldrich, Saint Louis, MO, USA) in the fibre assembly was inserted into the cap of the vial and was exposed to the headspace for 1 min. The methodology and selection of the fibre was based on previous studies of (Lu 2005). The fibre was then transferred to the GC injection inlet maintained at 240 °C and exposed to rapidly desorbed compounds onto the GC- IRMS column via a 15 s manual splitless injection. The sample were run on Zebron ZB-FFAP GC capillary column (length 30 m; internal diameter 0.25 mm; film thickness 0.25 µm; Phenomenex Inc., California, USA). The column temperature was programmed at 40 °C with a 5 min hold, ramped to 120 °C at 5 °C/min and ramped to 240 °C at 20 °C/min with a 2 min hold. Compounds eluting from column were oxidised to CO_2 via the oxidation furnace as described above.

2.18.6 Preparation of derivatised VFA samples for gas chromatography mass spectrometry

The supernatants obtained after centrifugation of 1.8 ml liquid fermentation samples that had been kept at –20 °C without internal standard added were thawed at room temperature (samples from experiment 2 and 3 in Chapter 4). The supernatant was again centrifuged at 21,000 *g* for 10 min at 4 °C, and 200 µl of this supernatant was transferred to 1.5 ml microfuge tubes and 100 µl concentrated HCl, 5 µl resazurin (blue dye; 0.1% w/v) and 800 µl diethyl ether were added. This was shaken vigorously and left it for 1 min at room temperature. The ether layer at the top layer was collected into a 2 ml microfuge tube. A further 800 µl diethyl ether was added to the aqueous phase remaining in the first tube, with repeated shaking and settling for 1 min. The ether layer was removed and added to the ether layer extracted in the first extraction. 800 µl of this ether solution was added to a crimp top GC vial and 100 µl derivatisation reagent (*N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide; Sigma-Aldrich, Saint Louis, MO, USA) was added. The preparations were heated for 20 min at 80 °C and then the vials kept at room temperature for 24 h before analysing their contents by GC as described by Richardson *et al.* (1989). One microlitre samples were run on a Shimadzu QP5050A mass spectrometer (Shimadzu Scientific Instruments (Oceania) Pty. Ltd, Auckland, New Zealand) equipped with a Shimadzu GC-17A and AOC-20i autosampler. The column used was a Zebron ZB-5ms (length 25 m; internal diameter 0.5 mm; film

thickness 25 μm; Phenomenex Inc., California, USA). Helium was used as the carrier gas at a flow rate of 1.5 ml/sec. The injector temperature was set at 250 °C, with a 1:20 split injection mode. The column temperature was programmed at 50 °C with a 4 min hold, ramped to 105 °C at 5 °C/min and ramped to 250 °C at 20 °C/min with a 4 min hold. The electron impact ionisation was employed and selected ion monitoring was conducted for the following ions (Table 2.13).

Table 2.13 Ions monitored with mass to charge ratio (m/z) of fragment ion.

No. of ^{13}C	0	1	2	3	4
Mass of fragment ion	M-57	M+1-57	M+2-57	M+3-57	M+4-57
		mass to charge ratio (m/z)			
Acetate	117	118	119		
Propionate	131	132	133	134	
Butyrate	145	146	147	148	149

The chosen ions represents the mass (M) of the derivatised compound minus the tertiary butyl fragment (mass 57) which represented the most abundant ion fragment retaining all carbons originating from the underivatised parent compound. Ion chromatograms were integrated and peak area ratios calculated relative to the M-57 base ion peak area for each compound. These ratios were then corrected for contributions from natural abundance and enriched species containing one or more less labelled carbons as described by the equations presented in the Appendix 1 (Table A.1; Wolfe & Chinkes 2004). The derived ratios were then used to calculate the mole percent excess of each labelled species as a percentage of total labelled and unlabelled species present.

2.19 *In vivo* trial

The components of the diet fed to sheep (wethers), sample collection from the animals and preparation of inhibitors administered to the animals are provided in the sections below. More details of the sheep are provided in Appendix 3 (Table A.3.1).

2.19.1 General purpose (GP) diet

One kilogram of GP diet contained 500 g hay, 290 g barley, 100 g soya bean meal, 100 g molasses, 3 g salt, 15 g minerals/vitamins and 55 g dicalcium/phosphates.

This is a conserved roughage diet formulated for general animal maintenance, growth and to fulfil nutritional requirements.

2.19.2 Preparation of inhibitors for *in vivo* trial

- Acetylene bolus: Due to the inability to compress acetylene gas, acetylene gas was slowly generated in the rumen *in situ* from a bolus containing calcium carbide which has been previously evaluated (M. Tavendale, personal communication). A single bolus consisted of 6.7 g barium sulfate ($BaSO_4$), 3.4 g iron powder (Fe), 1.4 g beeswax and 3.5 g calcium carbide (CaC_2). The beeswax was used to moderate the rate of reaction. The iron powder and barium sulfate were used to increase the density of the bolus and maintain the bolus below the surface of the rumen liquor. Calcium carbide reacts with water and releases acetylene into the rumen as described in equation (1):

  $$CaC_2 + H_2O \rightarrow C_2H_2 \text{ (acetylene)} + Ca(OH)_2 \text{ (calcium hydroxide)} \quad (1)$$

  A bolus with a payload to generate 500 ml of acetylene gas per day and overall bolus weight less than 8 g was administered. Prior to feeding, an acetylene generating bolus was inserted through the rumen fistula. The slow release bolus was suspended from the bung of the fistula in a nylon bag to prevent its regurgitation and to facilitate removal on a daily basis during the treatment period (5 days). The bolus was replaced with a new bolus every day and $BaSO_4$, Fe and wax residues from the previous bolus were removed.
- Chloroform: The chloroform treatment group received a chloroform-cyclodextrin paste added to a gelatine capsule and dosed directly to the rumen via the rumen fistula. The beta-cyclodextrin chloroform complex has been adapted from a previous study and evaluated in a sheep trial at AgResearch (M. Tavendale, personal communication; (May *et al.* 1995)). It was prepared by dissolving 60 g beta-cyclodextrin (food grade beta-cyclodextrin CTD Holdings Inc, Florida, USA) in 4 litre distilled water and adding 100 ml chloroform in a 10 litre Schott bottle. A white cyclodextrin chloroform inclusion complex precipitate was formed. The bottle was kept in a cold room for 7-8 days, then filtered to collect the precipitate which was washed with chilled water and stored at 4 °C until use. 1.8 g of paste (10 % w/w chloroform) was added to a

gelatine capsule, and one capsule was added into the fistula every morning for the five-day treatment period.

- Control bolus: The control group received a non-acetylene generating control bolus and beta-cyclodextrin containing no chloroform. A single non-acetylene generating control bolus consisted of 6 g barium sulfate, 3 g iron powder, 1.6 g beeswax and 4 g calcium carbonate. The overall bolus weighed less than 8 g when administered. Prior to feeding, a control bolus was inserted through the rumen fistula. The slow release bolus was suspended from the bung of the fistula in a nylon bag to prevent its regurgitation and to facilitate removal on a daily basis during the treatment period (5 days). Along with the non-acetylene generating control bolus, 1.8 g of cyclodextrin enclosed in a gelatine capsule was also dosed directly to the rumen via the rumen fistula to each sheep for the five-day treatment period.

2.19.3 Sample collection from animals

Nearly 200 ml rumen fluid was collected from each fistulated sheep to carry out *in vitro* experiments or other analysis. The samples were collected in thermos flasks to maintain the temperature to 39 °C. For future DNA extraction and analysis, 10 ml rumen fluid was collected in 50 ml plastic bottles and stored in a –85 °C freezer. Samples for VFA analysis were processed as described in Section 2.18.4.

2.20 Software used for statistical analysis of data

Data were analysed using one way ANOVA to compare treatment followed by Dunnett's multiple comparison with control using the Minitab software analysis tool (version 17; Minitab Inc., Pennsylvania, USA) or using Repeated measures ANOVA implemented in R software version 3.3.0 and packages such as *nlme* (and its *lme* function) and *predictmeans* (R Core Team 2016).

2.21 FTHFS cloning and analysis of FTHFS sequences

Total genomic DNA was extracted from 300 mg freeze-dried and homogenized rumen contents from control, acetylene- and chloroform-treated animals at day 1 (pre-inhibition period) and day 8 (after five days of treatment) using the PCQI

method described earlier. Genes encoding formyltetrahydrofolate synthetase (FTHFS) were then amplified using extracted DNA and the FTHFSf and FTHFSr primers and PCR protocol described in Table 2.12. Amplified FTHFS gene PCR products were run on agarose gel and clear bands confirmed. Then, these amplified FTHFS gene PCR products were cloned in *Escherichia coli* using the pGEM-T Easy vector system II (Promega, Madison, USA). The presence of inserts was confirmed by conducting colony PCR with primers GEM2945f and GEM189r and conditions described in Table 2.12. The colony PCR products were again run on agarose gels to confirm the presence of inserts by the presence of strong and clear bands. The samples were then sent to Macrogen Inc. (Seoul, Republic of Korea) for Sanger sequencing using the primers SP6 (5'-ATT TAG GTG ACA CTA TAG-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3').

FTHFS sequences obtained from Macrogen Inc. were imported into the ContigExpress package of Vector NTI (Vector NTI Advance 11, Invitrogen, Carlsbad, USA). The sequences were trimmed to remove poor end regions of the sequence. FTHFS sequences were then assembled, translated and aligned with ClustalW in the MEGA6.06 software package (Tamura *et al.* 2013), and the alignment manually checked and corrected. FTHFS sequences were then analysed using a profile hidden Markov model (HMM) implemented using the HMMER software package (Eddy 1998). A HMMER bit score was assigned to each sequence based upon its similarity to a given profile HMM, and the E-value, which provides the number of false-positive matches expected at or above the bit score, was calculated for each sequence using command the hmmsearch against HoF-HMM model. A detailed description of FTHFS sequencing and analysis is given by Henderson *et al.* (2010), and the parameters including the training set are described in Chapter 6.

Chapter 3

Identification of inhibitors that can be used to differentiate between methanogenesis and homoacetogenesis

3.1 Introduction

Enteric fermentation in ruminants results in the breakdown of carbohydrates to simple products such as volatile fatty acids, which are absorbed across the rumen wall and serve as a source of energy for the animal. Rumen fermentation also results in the formation of hydrogen, which is used by methanogens to produce methane. Methane is a potent greenhouse gas (GHG) that contributes to global warming, and also represents a 2-12% loss of feed gross energy for the animal (Johnson & Johnson 1995). Thus, it is of interest to inhibit methanogens to reduce both methane emissions and feed energy loss by ruminant livestock. Attempts have been made to reduce methane emissions from ruminants, either by directly inhibiting methanogens employing chemical inhibitors (Bauchop 1967, Martin & Macy 1985, Dumitru *et al.* 2003, Knight *et al.* 2011) or vaccines (Williams *et al.* 2009, Wedlock *et al.* 2013), or by reducing the formation of hydrogen that is converted to methane, by manipulating the diet (Moss *et al.* 1995, Martin 1998) or defaunation (Mosoni *et al.* 2011, Morgavi *et al.* 2012).

Inhibition of methane formation by inhibitors or vaccines administered to ruminants might lead to an accumulation of hydrogen in the rumen, which may slow down the feed fermentation due to accumulation of reducing equivalents (McAllister & Newbold 2008). The fate of hydrogen and the activity of alternative hydrogen utilisers in the rumen, which could use this hydrogen to form products that benefit the animal, are therefore of interest to understand the effects of methanogen inhibition. Homoacetogens, organisms that use carbon dioxide and hydrogen to produce acetate (Drake 1994, Schink 1994), are known to be present in the rumen (Leedle & Greening 1988, Henderson *et al.* 2010), but they are thought to be unable to compete with methanogens for hydrogen (Le Van *et al.* 1998, Nollet *et al.* 1998). If we want to investigate the activity of homoacetogens when methanogens are inhibited, a methanogen-specific inhibitor that does not inhibit homoacetogens and general rumen fermentation is needed. Such an inhibitor could be used experimentally to provide an insight into what happens when hydrogen is

no longer disposed of *via* methanogenesis, and to see if homoacetogenesis can represent a significant means of alternative hydrogen disposal. When screening for methanogen inhibitors, it is important to evaluate each inhibitor in a mixed microbial community (Ungerfeld *et al.* 2004), as microbes are in constant interaction with each other, and inhibiting one part of a microbial community might have severe effects on other parts. Prior to that, however, it is essential to study the effect of each inhibitor on individual methanogen strains in pure culture, and also on homoacetogens and other bacteria. A range of inhibitors has been tested against pure cultures of methanogens isolated from various environments and other bacteria (Table 3.1). However, to date, the effects and the efficacy of these against other rumen microbes have not been systematically assessed. In this chapter, the inhibitory effects of routinely used methanogenesis inhibitors (BES, acetylene and chloroform) was assessed over a range of concentrations, and against a variety of methanogen, homoacetogen and rumen bacterial cultures, to assess the specificity of these inhibitors.

As well as methanogen-specific inhibitors, it would be advantageous to identify a specific inhibitor of homoacetogens, in order to study the amount of rumen homoacetogenesis occurring in the rumen, and the significance of homoacetogenesis once methanogenesis is inhibited. The A-centre of the carbon monoxide dehydrogenase (CODH) enzyme, which mediates synthesis of acetyl-CoA from a methyl group plus carbon monoxide (CO) and CoA, is an excellent target for inhibition, since it plays a key role in acetyl-CoA synthesis *via* the Wood Ljungdahl pathway present in homoacetogenic bacteria (Ljungdahl 1986, Ragsdale *et al.* 1988, Ragsdale *et al.* 1990, Wood & Ljungdahl 1991, Kumar & Ragsdale 1995). Several chemical compounds have been described that inhibit the A-centre of the carbon monoxide dehydrogenase (CODH) enzyme (Ragsdale & Kumar 1996, Doukov *et al.* 2002). Therefore, known compounds that inhibit the carbon monoxide dehydrogenase (CODH) enzyme were tested for their inhibitory effects against homoacetogens. These were *n*-butyl isocyanide (Kumar & Ragsdale 1995), 1,10-phenanthroline (Shin & Lindahl 1992) and 5,5'-dithio-bis-(2-nitrobenzoic acid; Ragsdale & Wood 1985, Seravalli *et al.* 2003). Other inhibitors tested were fluoroacetate, which blocks acetate metabolism (Banat *et al.* 1981, Alperin & Reeburgh 1985, Chidthaisong & Conrad 2000), and rifampicin, an antibiotic that has been shown to inhibit acetogenic bacteria in acid bog soils without affecting

methanogenesis (Brauer *et al.* 2004, Sun *et al.* 2012). These reported chemical compounds (Table 3.1) were screened against a selection of methanogen and homoacetogen strains over a range of concentrations, in order to find a homoacetogen-specific inhibitor, as these inhibitors have not been previously tested against pure cultures of rumen methanogens or homoacetogens.

This chapter reports the screening of inhibitors against cultures of methanogens, homoacetogens and other rumen bacteria. This is a primary step towards finding specific inhibitors of methanogens and homoacetogens, which will be used for further *in vitro* (Chapters 4 and 5) and *in vivo* (Chapter 6) studies to allow alternative hydrogen utilisation pathways to become established in the sheep rumen *in vivo* and in sheep rumen fluid *ex vivo*, and to study the significance of homoacetogens in the absence of methanogens.

Table 3.1 Overview of inhibitor compounds screened against archaeal and bacterial cultures, and some selected inhibitors of homoacetogens.

Compounds	Proposed inhibition mechanism	Strain (and isolation source if known) or environment assessed	Reported outcomes	References
Inhibitors of methanogens				
2-bromoethanesulfonate (BES)	Structural analogue of coenzyme M	*Methanobrevibacter ruminantium* M1, *Methanosarcina mazei* CW3A, *Methanomicrobium mobile* BP (rumen)	Methanogens were inhibited at 250 μM concentration. *Methanosarcina mazei* CW3A appeared to develop resistance to BES from day 6, even at 250 μM concentration. *Methanobrevibacter ruminantium* M1 was inhibited, but the effects wore off by day 6 at 50 μM	Taylor & Wolfe (1974), Gunsalus *et al.* (1978), Ungerfeld *et al.* (2004)
Acetylene	Affects ATP synthesis, proton movement and maintenance of pH gradient	*Methanobacterium bryantii* M.o.H (anaerobic digestor), *Methanothermobacter thermoautotrophicus* ΔH (sewage sludge), *Methanobacterium* sp. G2R, *Methanosarcina barkeri* (unnamed strain; anaerobic sewage digestor), *Methanospirillum hungatei* JF1 (sewage sludge), *Methanospirillum hungatei* GP1 (anaerobic digestor). Selected archaeal *Halobacterium* spp. and bacteria	Methane formation by methanogens was inhibited at a concentration of 65 μM. Non-methanogenic archaea and bacteria were not inhibited, even at 325 μM	Sprott *et al.* (1982)

Compounds	Proposed inhibition mechanism	Strain (and isolation source if known) or environment assessed	Reported outcomes	References
	Inhibits nickel-containing hydrogenase	*Methanococcus voltae* PS (mud), *Methanosarcina* sp. MST-A1 (digestor sludge) and selected sulfate-reducing bacteria	Hydrogenases isolated from these microbes were inhibited by various concentrations	He *et al.* (1989)
Chloroform	Inhibits methyl coenzyme M reductase	*Methanobrevibacter ruminantium* S231 (rumen), *Methanobacterium bryantii* M.o.H (mud)	Methane formation was inhibited 50% in the presence of 8 μM chloroform	Prins *et al.* (1972), Gunsalus & Wolfe (1978)
Reported inhibitors of homoacetogenesis				
Rifampicin	Inhibits RNA polymerase	*Methanobrevibacter smithii* PS, *Methanobrevibacter smithii* F1, *Methanobrevibacter smithii* ALI, *Methanobrevibacter smithii* B181, *Methanobrevibacter oralis* Milano, *Methanosphaera stadtmanae* MCB-3, *Methanomassiliicoccus luminyensis* B10 (human faeces)	Methanogens were inhibited at a concentration ≥121.5 μM	Dridi *et al.* (2011)
		Bacteria such as *Escherichia coli*, *Bacillus subtilis* W 23, *Micrococcus luteus*, *Staphylococcus aureus*	Bacteria showed inhibition in agar diffusion tests	Hilpert *et al.* (1981)

Compounds	Proposed inhibition mechanism	Strain (and isolation source if known) or environment assessed	Reported outcomes	References
		Escherichia coli, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Neisseria* spp.	Bacteria showed inhibition in agar diffusion tests. Minimum inhibitory concentrations ranged from 0.005 to 19.4 μM for different bacteria	Andrews (2001)
		Bog soil	Inhibited acetogenesis at 12.2 μM	Brauer *et al*. (2004)
		Peat soil	Inhibited H_2-consuming acetogenic bacteria at 12.2 μM	Sun *et al*. (2012)
1,10-Phenanthroline	Inhibits carbon monoxide/acetyl-CoA exchange activity	Purified carbon monoxide dehydrogenase (CODH) enzyme from *Moorella thermoaceticum* VPI 12954	Carbon monoxide/acetyl-CoA exchange activity of carbon monoxide dehydrogenase (CODH) was lost in the presence of 1 mM of 1,10-phenanthroline	Shin & Lindahl (1992)
5,5'-Dithio-bis-(2-nitrobenzoic acid)	Inhibits exchange reaction between carbon monoxide and the carbonyl group of acetyl-CoA	Purified carbon monoxide dehydrogenase (CODH) enzyme from *Moorella thermoaceticum* VPI 12954	100% inhibition of carbon monoxide to CO_2 reactions and 93% inhibition of exchange reaction at 10 mM concentration	Ragsdale & Wood (1985)
n-Butyl isocyanide (*n*-BIC)	Structural analogue of carbon monoxide for carbon monoxide dehydrogenase (CODH) enzyme	Purified carbon monoxide dehydrogenase (CODH) enzyme from *Moorella thermoaceticum* VPI 12954	Reduced A, B and C centres of carbon monoxide dehydrogenase (CODH) at 0.3 mM	Kumar & Ragsdale (1995)

Compounds	Proposed inhibition mechanism	Strain (and isolation source if known) or environment assessed	Reported outcomes	References
Fluoroacetate	Blocks acetate metabolism	Sediment	Inhibited acetate metabolism by sulfate-reducing bacteria and methanogens at 5 mM or 10 mM	Banat *et al.* (1981), Alperin & Reeburgh (1985)
		Rice field soil	Inhibited acetoclastic methanogenesis, acetate consumption and sulfate reduction at 1 mM	Chidthaisong & Conrad (2000)

3.2 Objectives

The aims of the work described in this chapter were to:

- identify inhibitors specific for methanogens;
- identify inhibitors specific for homoacetogens;
- to test a range of inhibitor concentrations against a selection of rumen-relevant microbes, to ensure that these rumen-microbes are not adversely affected;
- determine suitable inhibitor concentrations to quantify rumen microbial homoacetogenesis and methanogenesis *in vitro* (Chapters 4 and 5) and *in vivo* (Chapter 6).

3.3 Summary of materials and methods

The inhibitory effects of ten compounds were assessed against eight methanogen, three homoacetogen and six bacterial strains (Tables 3.2 and 3.3). Briefly, and unless otherwise stated, microbes were cultured in RM02 medium (Kenters *et al.* 2011) using the Hungate technique (Bryant 1972, Macy *et al.* 1972) and maintained at 39 °C with shaking (200 rpm, Infors HT Labotron, Bottmingen, Switzerland) in the dark. For methanogens, RM02 medium was supplemented with clarified rumen fluid (Kenters *et al.* 2011) and vitamin solution (Janssen *et al.* 1997), 20 mM acetate, 20 mM methanol, and 60 mM formate, and maintained under a H_2/CO_2 (80:20 v/v) atmosphere. Two methanogenic strains were incubated in BY medium. These were *Methanomassiliicoccales* isolate ISO4-H5, due to its inability to grow in RM02 medium, and *Methanobrevibacter* sp. D5, which grew much slower in the RM02 medium than in BY (Joblin *et al.* 1990). For these two strains, acetate, methanol, formate and vitamin solution were added to BY as described above. The BY medium for ISO4-H5 was supplemented with 10 μM coenzyme M (Sigma-Aldrich, Saint Louis, MO, USA) and filtered *Succinivibrio dextrinosolvens* H5 culture supernatant, which is required for its growth. *Succinivibrio dextrinosolvens* H5 cultures that were source of the spent medium were grown in BY medium supplemented with 1% (w/v) pectin. The spent medium was filtered through a 0.22 μm pore size syringe filter into the ISO4-H5 culture tubes, so that 1 ml of H5 culture filtrate was added to 9 ml of culture medium for growing ISO4-H5. For rumen bacteria, RM02 medium was supplemented with clarified rumen fluid (Kenters *et*

al. 2011; containing 0.34 g each of D-glucose and D-cellobiose, 0.30 g each of D-xylose and L-arabinose, 0.88 ml of Na L-lactate syrup, containing 50% Na L-lactate), 2 g each of casamino acids, Bacto-peptone and yeast extract per 100 ml of clarified rumen fluid) and vitamin solution (Janssen *et al.* 1997), and maintained under a CO_2 atmosphere. Homoacetogens were grown in RM02 supplemented with clarified rumen fluid (Kenters *et al.* 2011) and vitamin solution (Janssen *et al.* 1997), and either 5 mM glucose maintained under CO_2 atmosphere or under a H_2/CO_2 (80:20 v/v) atmosphere. Inhibitor (and non-inhibited control) stock solutions were prepared under anaerobic conditions and added to the culture media at different concentrations at the start of the experiments (Tables 3.2 and 3.3). Non-inhibited control tubes rececived equal amounts of filter-sterilized distilled water or media used to prepare the inhibitor stock solution. Acetylene concentrations were achieved by adding a defined amount of gas to the headspace of the culture tubes, after having calculated the amount that would dissolve at the incubation temperature, and correcting for the partitioning of gas between the headspace and liquid phase.

To assess the effects of potential inhibitors, cultures in the early stationary growth phase were used to inoculate culture tubes containing fresh media with and without inhibitors. The growth of these newly-inoculated cultures was followed over time by following optical densities at 600 nm in a spectrophotometer (Spectronic 200, Thermo Fischer Scientific, Mumbai, India) by inserting the culture tubes directly into the spectrophotometer approximately every 12 h over a period of 2 days, until the growth reached the stationary phase. Methane production was used to assess the growth of methanogens. The amount of methane formed was measured using gas chromatography (GC), 7 days after inoculating the cultures. Headspace gas, 0.3 ml, from each methanogen culture tube was injected into an Aerograph 660 (Varian Associates, Palo Alto, CA, USA) equipped with a Porapak Q 80/100 mesh column (Waters Corporation, Milford, MA, USA) and a thermal conductivity detector at 100 °C. The column was operated with N_2 as the carrier gas at room temperature. Prior work with these methanogens, including preparing cultures to use as inocula, showed that 7 days was sufficient time for them to reach stationary phase. Compounds were deemed to be inhibitory against rumen bacteria and homoacetogens if the optical density of the culture was less than 10% compared to the non-inhibited control. A compound was deemed to be inhibitory towards a

methanogen if no methane was produced, when compared with the non-inhibited control incubation of the same methanogen. Experiments were carried out in triplicate. Further details on the methodology can be found in Chapter 2.

3.4 Results and discussion

3.4.1 Chloroform

The inhibitory effects of a range of chloroform concentrations (5 to 2000 μM) were assessed on pure cultures of methanogens as well as homoacetogens (grown with glucose or H_2+CO_2) and other rumen bacteria (Table 3.2). All the methanogen strains tested were inhibited at a very low concentration (5 μM), and so chloroform proved to be a very effective inhibitor. Homoacetogens grown with H_2+CO_2 were partially inhibited by chloroform at concentrations greater than 50 μM and completely inhibited at 2000 μM. Homoacetogen strain *Eubacterium limosum* SA11 grown with glucose was also very sensitive to chloroform and was completely inhibited at 50 μM concentration, whereas *Blautia* spp. Ser5 and Ser8 grown with glucose were completely inhibited at 2000 μM but not at 1000 μM. Chloroform inhibited the growth of all other tested bacterial strains at 2000 μM.

3.4.2 2-bromoethanesulfonate (BES)

Methane production by *Methanobrevibacter millerae* SM9, *Methanobrevibacter ruminantum* M1 and *Methanosphaera* sp. ISO3-F5 was completely inhibited by 5 μM BES. In contrast, *Methanobrevibacter* sp. D5, *Methanobacterium formicicum* BRM9 and *Methanomassiliicoccales* isolate ISO4-H5 were inhibited at higher concentrations of BES (2000, 1000 and 10 μM, respectively; Table 3.2). BES seemed to have no effect on the growth of homoacetogens in the presence of either H_2+CO_2 or glucose, even at a concentration as high as 10 mM. Growth of other bacterial strains was not affected by BES concentration as high as 10 mM.

3.4.3 Acetylene

Acetylene inhibited methane production by methanogen strains at concentrations ranging from 5 to 1000 μM dissolved acetylene (Table 3.2). *Methanobrevibacter millerae* SM9, *Methanobrevibacter ruminantum* M1 and *Methanosphaera* sp. ISO3-F5 were inhibited at concentrations as low as 5 to 10 μM, whereas *Methanobacterium formicicum* BRM9 and *Methanobrevibacter* sp. D5 were inhibited at acetylene concentrations of 50 μM and 100 μM, respectively.

Interestingly, *Methanomassiliicoccales* isolate ISO4-H5 was less sensitive to acetylene, and a very high concentration of 1000 μM was needed before it no longer produced methane (although concentrations between 100 and 1000 μM were not tested). Acetylene did not affect the growth of homoacetogens in the presence of glucose or H_2+CO_2, and had no effect on growth of other bacterial strains, even at a concentration of 1000 μM.

3.4.4 Inhibitors of homoacetogens

The effects of various potential inhibitors of homoacetogens (Table 3.1) were assessed against pure cultures of homoacetogens and methanogens, as these inhibitors have not been previously screened against rumen methanogens and against homoacetogens grown with glucose (Table 3.3). Fluoroacetate did not inhibit the growth of homoacetogens even at 5 mM, but some methanogens were partially inhibited at this concentration. *N*-Butyl isocyanide partially inhibited the growth of the homoacetogens *Blautia* spp. Ser5 and Ser8, and completely inhibited *Eubacterium limosum* SA11 at 5 mM, and it inhibited growth of two of the methanogens tested. High concentration of phosphate (10 mM and 100 mM) had no effect on growth of homoacetogens. 1,10-Phenanthroline and 5,5'-dithio-bis-(2-nitrobenzoic acid) inhibited homoacetogens at 1 mM and 5 mM, and also inhibited growth of methanogens at this concentration. Rifampicin was the only compound that showed some potential as a specific homoacetogen inhibitor at 6 and 60 mM, in that it inhibited homoacetogens, but not methanogens.

Table 3.2 Growth of selected rumen methanogens, homoacetogens (grown with either glucose or H_2 + CO_2) and rumen-relevant bacteria in the presence of known inhibitors of methanogens.

	Inhibitor and concentration (μM)																								
	Chloroform							BES									Acetylene								
Isolate	0	5	10	20	50	1000	2000	0	5	10	20	50	1000	2000	5000	10000	0	5	10	20	50	100	1000		
Methanogens																									
Methanobrevibacter millerae SM9	+	–	–	–	–	–	–	+	–	–	–	–	–	ND	ND	ND	+	+	–	–	–	–	–		
Methanobrevibacter sp. D5	+	–	–	–	–	–	–	+	ND	ND	ND	ND	+	–	ND	ND	+	+	+	+	+	–	–		
Methanobrevibacter ruminantium M1	+	–	–	–	–	–	–	+	–	–	–	–	–	ND	ND	ND	+	–	–	–	–	–	–		
Methanobacterium formicicum BRM9	+	–	–	–	–	–	–	+	+	+	+	+	–	ND	ND	ND	+	+	+	+	–	–	–		
Methanosphaera sp. ISO3-F5	+	–	–	–	–	–	–	+	–	–	–	–	–	ND	ND	ND	+	–	–	–	–	–	–		
Methanomassiliicoccales isolate ISO4-H5	+	–	–	–	–	–	–	+	+	–	–	–	–	ND	ND	ND	+	+	+	+	+	+	–		
Homoacetogens																									
Blautia sp. Ser5 (glucose)	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Blautia sp. Ser5 (H_2+CO_2)	+	+	+	+	+	(+)	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Blautia sp. Ser8 (glucose)	+	+	+	+	+	(+)	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Blautia sp. Ser8 (H_2+CO_2)	+	+	+	+	+	(+)	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Eubacterium limosum SA11 (glucose)	+	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Eubacterium limosum SA11 (H_2+CO_2)	+	+	+	+	(+)	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Other bacteria																									
Butyrivibrio proteoclasticus B316	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Prevotella ruminicola 23	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Ruminococcus flavefaciens FD1	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Selenomonas ruminantium GA-192	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Fibrobacter succinogenes S85	+	+	+	+	+	(+)	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Bacteroides sp. Ga6A1	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		

+, Growth with no inhibition and comparable to non-inhibited control; –, no growth; (+), growth less than non-inhibited control (around 50%); ND, not determined.

Table 3.3 Growth of selected rumen methanogens and homoacetogens in the presence of potential inhibitors of homoacetogens.

	Inhibitor and concentration (μM)													
	Fluoroacetate		***n*-Butyl isocyanide**		**Phosphate**		**Rifampicin**		**1,10-Phenanthroline**		**5,5'-dithio-bis-(2-nitrobenzoic acid)**			
Isolate	20	5000	1000	5000	10000	100000	6	60	1000	5000	1	100	1000	5000
Homoacetogens														
Blautia sp. Ser5 (glucose)	+	+	+	(+)	+	+	–	–	–	–	+	+	–	–
Blautia sp. Ser8 (glucose)	+	+	+	(+)	+	+	–	–	–	–	+	+	–	–
Eubacterium limosum SA11 (glucose)	+	+	(+)	–	+	+	–	–	–	–	+	+	–	–
Methanogens														
Methanobrevibacter sp. YML1	+	(+)	ND	ND	ND	ND	+	+	ND	ND	ND	ND	ND	ND
Methanosphaera sp. ISO3-F5	+	(+)	ND	ND	ND	ND	+	+	–	–	–	–	–	–
Methanobrevibacter sp. AbM4	ND	ND	–	–	+	+	+	+	–	–	–	–	–	–
Methanobrevibacter millerae SM9	ND	ND	–	–	+	+	+	+	–	–	ND	ND	ND	ND

+, Growth with no inhibition and comparable to non-inhibited control; –, no growth, (+), growth less than non-inhibited control (around 50%); (-), little growth (less than 75% of non-inhibited control); ND, not determined.

3.4.5 Discussion

Three known inhibitors of methanogens (chloroform, BES and acetylene) were tested at various concentrations against selected strains of methanogens, homoacetogens and other bacteria. Chloroform is a halogenated hydrocarbon that has been reported to inhibit methyl coenzyme M reductase, a key enzyme involved in last step of methanogenesis (Gunsalus & Wolfe 1978). Chloroform has also been studied and used as an inhibitor of methanogenesis during *in vitro* rumen fluid incubation (Bauchop 1967, Yang & Speece 1986), as well as in ovine (Lanigan 1972) and cattle rumens (Knight *et al.* 2011). Pure culture studies conducted by Prins *et al*. (1972) resulted in 50% inhibition of the rumen isolate *Methanobrevibacter ruminantium* S231 and the mud isolate *Methanobacterium bryantii* M.o.H by 8 μM chloroform. In the present study all the methanogen strains screened were completely inhibited by 5 μM chloroform. Chloroform at concentrations of 20 μM and 50 μM has been reported to inhibit homoacetogens (*Acetobacterium woodii* WB1, isolated from mud; *Sporomusa acidovorans* Mol, isolated from alcohol-distillery plant effluent) growing with H_2 (Scholten *et al.* 2000). In the pure culture studies reported in this chapter, homoacetogens grown on H_2+CO_2 were partially inhibited at chloroform concentrations greater than 50 μM, and *Eubacterium limosum* SA11 (grown on glucose or H_2+CO_2) was found to be most sensitive to chloroform. Although chloroform has been extensively used in the published literature as a methanogenesis inhibitor, it could not be reliably used as a specific methanogen inhibitor as it also inhibits homoacetogens at concentrations that are not much greater than those that inhibit methanogens.

BES, a structural analogue of coenzyme M (CoM), inhibits the methyl transfer reaction during methane formation from carbon dioxide and hydrogen (Taylor & Wolfe 1974, Gunsalus *et al.* 1978), and has been used to inhibit methanogenesis in a variety of environments, including a thermophilic anaerobic digester (Zinder *et al.* 1984), rice root systems (Chidthaisong & Conrad 2000), the rumen (Immig *et al.* 1996, Ungerfeld *et al.* 2004) and soil (Wüst *et al.* 2009). The effective concentration seems to vary in different environments. In a thermophilic anaerobic digester 50 mM BES inhibited methanogens (Zinder *et al.* 1984), in rice root systems 10 mM BES inhibited methanogens (Chidthaisong & Conrad 2000), and in soil 5 mM to 20 mM inhibited methanogens (Wüst *et al.* 2009). However,

the levels of inhibition reported here are broadly comparable with those of a previous study with a pure culture, where methane production by *Methanobrevibacter ruminantum* M1 was reduced by 14-34% at 10 μM BES, 70-90% at 50 μM BES, and completely inhibited at 250 μM BES at days 4 and 6 (Ungerfeld *et al.* 2004). Therefore, a decision was made to use 3 mM BES for further *in vitro* studies, as the growth of methanogens tested was inhibited at this concentration, whereas growth of homoacetogens and other bacteria was not affected.

Acetylene affects ATP synthesis, proton movement, and maintenance of pH gradients (Sprott *et al.* 1982), and has been reported to inhibit methanogens in waste biodegradation (Zhao *et al.* 2009), anaerobic paddy soils (Raimbault 1975) and marine sediments (Oremland & Taylor 1975). In pure cultures studies reported by Sprott *et al*. (1982), all the methanogen cultures tested were inhibited at 65 μM, while the growth of extremely halophilic (non-methanogenic) archaea and bacteria was not affected by acetylene even at a 325 μM concentration (Sprott *et al.* 1982). Oremland and Taylor (1975) reported complete inhibition of methane production at 0.5 mM to 8.3 mM of acetylene in marine sediments (Oremland & Taylor 1975). However, potential inhibitory effects of acetylene at different concentrations against a variety of microbes have not yet been studied in detail. Data from these previous studies are comparable with the present study, where the effective inhibitory acetylene concentration against methanogens varied between 5 to 100 μM, whereas no effect on other bacteria was observed, even at a concentration of 1000 μM. Acetylene has been reported to inhibit hydrogenase activity from *Methanococcus voltae* PS and *Methanosarcina* MST-A1 (He *et al.* 1989). For this reason, growth of homoacetogens using H_2+CO_2 as substrates was also assessed, to ensure acetylene did not inhibit hydrogen use by homoacetogens. Acetylene showed no inhibitory effects against homoacetogens grown in the presence of H_2+CO_2 or glucose. Based on these findings, acetylene at concentrations greater than 100 μM was selected to inhibit methanogens during *in vitro* and *in vivo* trials presented in this thesis.

Some inhibitors that were selected for screening against homoacetogens (Table 3.1) were reported to inhibit the carbon monoxide dehydrogenase/acetyl-CoA synthetase (CODH/ACS) complex present in homoacetogens (Ragsdale *et al.*

1988, Ragsdale *et al.* 1990, Wood & Ljungdahl 1991). These were *n*-butyl isocyanide (Kumar & Ragsdale 1995), 1,10-phenanthroline (Shin & Lindahl 1992) and 5,5'-dithio-bis-(2-nitrobenzoic acid; Ragsdale & Wood 1985, Seravalli *et al.* 2003). In addition, fluoroacetate was tested, as it blocks acetate metabolism, when converted to fluorocitrate, which inhibits aconitase during the tricarboxylic cycle, and therefore blocks acetate metabolism by sulfate-reducing bacteria and methanogens (Banat *et al.* 1981, Alperin & Reeburgh 1985, Chidthaisong & Conrad 2000). Most of the potential homoacetogen-specific inhibitors screened in the present study would be unsuitable for the purpose of studying alternative hydrogen utilisation by homoacetogens, as they either did not inhibit homoacetogens (fluoroacetate, phosphate), also inhibited methanogens (*n*-butyl isocyanide, 5,5'-dithio-bis-(2-nitrobenzoic acid), or inhibited both homoacetogens and methanogens (1,10-phenanthroline). Rifampicin has been shown to inhibit acetogenic bacteria in acid bog soils, without affecting methanogenesis (Brauer *et al.* 2004, Sun *et al.* 2012). Rifampicin, when tested against pure cultures of human methanogens at concentrations greater than or equal to 121.5 μM inhibited the growth of methanogens (Dridi *et al.* 2011). In the present study rifampicin was tested at much lower concentrations (6 μM and 60 μM), and it did not inhibit methane production by methanogens, but it inhibited homoacetogens. However, rifampicin is a known antibiotic that inhibits bacterial RNA polymerase and is reported to inhibit bacteria (Hilpert *et al.* 1981). Rifampicin is known to inhibit bacteria at as low as 0.005 μM concentration, though this can vary between different species (Table 3.1; Andrews 2001), meaning it is likely to adversely affect rumen fermentation. Therefore, a suitable specific inhibitor of homoacetogens that did not inhibit methanogens or affect rumen fermentation was not identified.

3.5 Summary and perspectives

The evaluation of different inhibitors in this chapter showed that BES and acetylene are highly specific inhibitors of methanogens that did not affect the growth of homoacetogens and other rumen bacteria tested. However, given that some methanogens reportedly develop resistance to BES (Ungerfeld *et al.* 2004), this inhibitor is unlikely to be a suitable for reduction of methane production over a prolonged period of time. A previous study, where BES was used to induce reductive acetogenesis in the rumen of one sheep, seems to confirm this, as

methanogenesis returned to pre-inhibition levels after only four days, presumably due to the adaptation of methanogens to BES (Immig *et al.* 1996). However, BES is nonetheless suitable as a tool to investigate short-term effects that inhibition of methanogenesis has on fermentation patterns. For this reason, BES was used to measure homoacetogenesis in absence of methanogens in short-term *in vitro* assays developed in Chapter 4, and used in Chapters 5 and 6. Acetylene can inhibit methane production by rumen contents *in vitro* (Elleway *et al.* 1971) and from sheep (M. Tavendale, personal communication). For this reason, acetylene was the inhibitor chosen for longer *in vitro* and *in vivo* trials (Chapters 5 and 6).

No suitable inhibitor specific for homoacetogens was identified. Although rifampicin was found to inhibit homoacetogens, it is known to be a general bacterial inhibitor that might affect rumen fermentation (Hilpert *et al.* 1981). Chloroform was found to be very effective against homoacetogens, but also strongly affected the growth of methanogens, therefore it was deemed unsuitable to study the specific inhibition of either group. However, as chloroform inhibits both methanogens and homoacetogens, it was decided to use chloroform to indirectly assess homoacetogenesis. An inhibitor of both methanogens and homoacetogens would be beneficial as an additional control in the studies planned for this thesis, to examine the potential importance of acetate formation via homoacetogenesis in the absence of methanogenesis. Thus, chloroform was used at a concentration of 100 μM in the *in vitro* (mixed culture) studies, to see if it inhibits both methanogens and homoacetogens as seen in earlier studies by Scholten *et al*. (2000), and whether can act as an additional control. To achieve this, fermentations where 1) methanogenesis (e.g., with BES or acetylene) or 2) methanogenesis and homoacetogenesis (e.g., with chloroform) were inhibited were compared, overcoming the issue that no specific inhibitor of homoacetogenesis was identified. The selected inhibitors could now be used to assess the impact of inhibiting methanogenesis in the *in vitro* rumen fermentation, and to determine to what extent homoacetogenesis replaces methanogenesis as a means of hydrogen utilisation.

Chapter 4

Development of an assay to measure homoacetogenesis by *in vitro* incubation of rumen fluid

4.1 Introduction

Inhibiting methane production from ruminants by direct inhibition of methanogens will alter the dynamics of hydrogen and electron flow in the rumen. For example, it has been suggested that hydrogen accumulation in the absence of methanogens, may decrease the rate of feed fermentation (Wolin 1979, McAllister & Newbold 2008). It would be advantageous to divert the hydrogen into the production of metabolites that can potentially serve as energy sources for the animal, while at the same time disposing of the hydrogen. Homoacetogenic bacteria (homoacetogens) use four moles of H_2 and two moles of CO_2 to generate acetate ($2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$; Drake 1994, Schink 1994). Homoacetogens are known to occur in the rumen (Leedle & Greening 1988, Henderson *et al.* 2010), but are unable to compete with methanogens for hydrogen (Morvan *et al.* 1994, Joblin 1999, McAllister & Newbold 2008), mainly because the energy yield of their metabolism of hydrogen is lower than that from metabolism of hydrogen to methane (Thauer *et al.* 1977, Cord-Ruwisch *et al.* 1988). However, it has been postulated that homoacetogens could play a role in hydrogen utilisation and disposal when methanogens are inhibited (Joblin 1999, Fonty *et al.* 2007).

The use of chemical inhibitors for experimental purposes can provide useful insights into microbial processes occurring in the rumen and could be used to determine the contribution of homoacetogenesis to hydrogen or electron use in the absence of methanogenesis. In Chapter 3, specific inhibitors of methanogens (BES and acetylene), and of both methanogens and homoacetogens (chloroform) were identified. One of the aims of the work described in this chapter was to determine whether these inhibitors are effective *in vitro* (mixed culture) against methanogens at the concentrations selected, and to measure the effects of inhibiting methanogenesis on *in vitro* rumen fermentation products. The major aim was to develop an assay to measure homoacetogenesis in the presence and absence of methanogenesis during short-term *in vitro* incubation of rumen fluid. For this, a

sensitive and accurate methodology using labelled CO_2 was used to distinguish acetate derived from carbohydrate fermentation from acetate derived from homoacetogenesis. Similar approaches have been used previously. The formation of acetate from CO_2 by incorporation of $^{14}CO_2$ into acetate was measured in caecal contents of rodents and in bovine rumen fluid (Prins & Lankhorst 1977), termite guts (Breznak & Switzer 1986, Tholen & Brune 1999), and in peat soil samples (Ye *et al.* 2014). Incorporation of $^{13}CO_2$ into acetate was used to estimate homoacetogenic activity in *in vitro* incubations with 100% H_2, using rumen contents from new born lambs that had been inoculated with homoacetogens (Morvan *et al.* 1994) or during incubation of bovine rumen contents with and without addition of the homoacetogen *Acetitomaculum ruminis* 1904A or the methanogen inhibitor BES (Le Van *et al.* 1998). The principles of these methods were used in the experiments described here and in the subsequent chapters, to estimate homoacetogenic activity in rumen contents in the presence and absence of methanogenesis, without adding homoacetogens or artificially elevating the hydrogen partial pressure.

Methods to measure homoacetogenesis were developed in this chapter to measure the incorporation of $^{13}CO_2$ (added as $NaH^{13}CO_3$) into ^{13}C-acetate over time in *in vitro* rumen fluid incubations, in the presence and absence of selected inhibitors of methanogens and homoacetogens. The incorporation of stable ^{13}C isotope into different products was analysed using gas chromatography-isotope ratio mass spectrometry (GC-IRMS). In addition to incorporation into acetate, ^{13}C-label can be expected to appear in other products, especially propionate (Godwin *et al.* 2014). VFA inter-conversion has been reported to occur during infusion of labelled VFA *in vivo*, with label appearing in one or more other VFA (Leng & Brett 1966, Glinsky *et al.* 1976, Sharp *et al.* 1982, Bruce *et al.* 1987, Sutton *et al.* 2003, Ungerfeld & Kohn 2006, Nolan *et al.* 2014). It might therefore be that label from propionate would find its way into acetate via inter-conversion. Therefore, *in vitro* experiments with the addition of $^{13}C_3$-propionate, 2-$^{13}C_1$-propionate, $^{13}C_2$-acetate and $^{13}C_4$-butyrate were carried out to confirm that measured homoacetogenesis was "real" and not an artefact of VFA interchange. Then, to avoid homoacetogenesis being over or under-estimated, the measured incorporation rates of the label were corrected for $^{13}CO_2$ being incorporated into ^{13}C-acetate via VFA interchange.

4.2 Objectives

Experiment 1. Develop an *in vitro* assay to measure homoacetogenesis

Rumen contents were incubated in the presence and absence of the inhibitors, BES or chloroform and $^{13}CO_2$ tracer (added as $NaH^{13}CO_3$). Methane, hydrogen and total gas formation were measured, the equilibrium between headspace and dissolved CO_2 was followed, and estimates of homoacetogenesis were made, to

- determine the impact of inhibitors on *in vitro* rumen fermentation
- determine the optimal time for collecting samples to measure homoacetogenesis and other fermentation variables such as volatile fatty acids (VFA)
- determine the effect of addition of stable isotope $NaH^{13}CO_3$ on *in vitro* rumen fermentation
- follow the incorporation of $^{13}CO_2$ into ^{13}C-acetate and other VFA
- determine the effect of addition of inhibitors on homoacetogenesis.

Experiment 2. Determine the effects of addition of large concentrations of VFA on *in vitro* rumen fermentation

To study VFA inter-conversions, large concentrations of VFA had to be added to *in vitro* fermentations. In this experiment, the aim was to

- determine what the impact of these additions was on *in vitro* gas and VFA production.

Experiment 3. Measure VFA inter-conversion from propionate to acetate and butyrate

Rumen contents were incubated in the presence and absence of BES or chloroform and tracer substrate ($^{13}C_3$-propionate or 2-$^{13}C_1$-propionate), to determine

- the fractional amount of $^{13}C_3$-propionate that is converted to $^{13}C_1$-acetate or $^{13}C_2$-acetate and $^{13}C_1$-butyrate, $^{13}C_2$-butyrate, $^{13}C_3$-butyrate or $^{13}C_4$-butyrate
- the fractional amount of 2-$^{13}C_1$-propionate that is converted to $^{13}C_1$-acetate or $^{13}C_2$-acetate and $^{13}C_1$-butyrate, $^{13}C_2$-butyrate, $^{13}C_3$-butyrate or $^{13}C_4$-butyrate.

Experiment 4. Measure VFA inter-conversion between acetate, propionate and butyrate

Rumen contents were incubated in the presence and absence of one of the tracer substrate ($^{13}C_2$-acetate, $^{13}C_3$-propionate, or $^{13}C_4$-butyrate) to determine VFA inter-conversion, and to

- measure the fractional amount of $^{13}C_2$-acetate that is converted to $^{13}C_1$-propionate, $^{13}C_2$-propionate or $^{13}C_3$-propionate and $^{13}C_1$-butyrate, $^{13}C_2$-butyrate, $^{13}C_3$-butyrate or $^{13}C_4$-butyrate
- measure the fractional amount of $^{13}C_3$-propionate that is converted to $^{13}C_1$-acetate or $^{13}C_2$-acetate and $^{13}C_1$-butyrate, $^{13}C_2$-butyrate, $^{13}C_3$-butyrate or $^{13}C_4$-butyrate
- measure the fractional amount of $^{13}C_4$-butyrate that is converted to $^{13}C_1$-acetate or $^{13}C_2$-acetate and $^{13}C_1$-propionate, $^{13}C_2$-propionate or $^{13}C_3$-propionate
- correct the amount of acetate formed from $^{13}CO_2$ by homoacetogenesis for any labelling that results from VFA inter-conversion.

4.3 Summary of materials and methods

An overview of the short-term assay used to develop the methodology and determine the effects of addition of BES or chloroform and of $NaH^{13}CO_3$ *in vitro*, and to measure homoacetogenesis, is provided in Figure 4.1 (Experiment 1). The short-term *in vitro* experiments used to measure the effect of addition of large amounts of VFA on *in vitro* rumen fermentation, and to measure VFA inter-conversions are described in Figures 4.2, 4.3 and 4.4 (Experiments 2, 3 and 4).

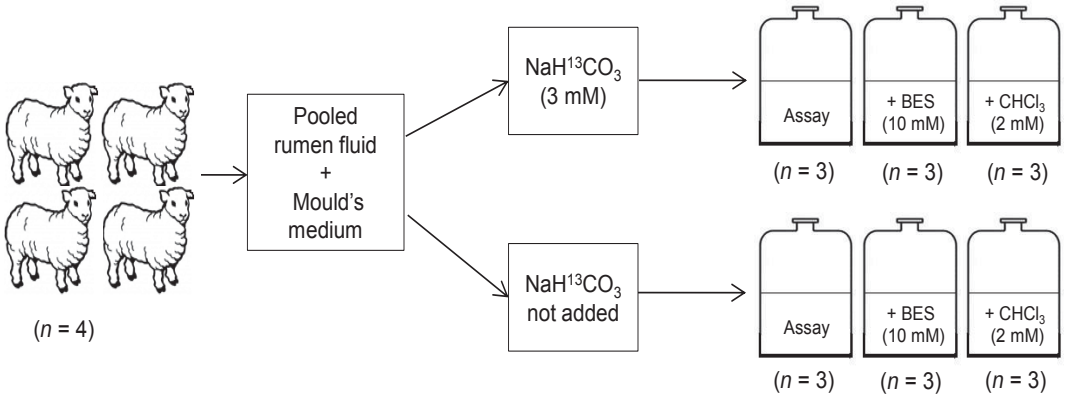

Figure 4.1 Experiment 1. Overview of the short-term *in vitro* assay used to measure homoacetogenesis. Methane, hydrogen and total gas production were continuously measured using gas chromatography (GC). Liquid (2 ml) and headspace gas (1 ml) samples were collected every two hours to measure VFA concentrations and the ratios of $^{13}C/^{12}C$ in dissolved (CO_2) and headspace gas (CO_2 and CH_4). Liquid samples collected following 8 hours of incubation were used to measure ^{13}C-labelled acetate for calculation of the amount of acetate formed by homoacetogenesis.

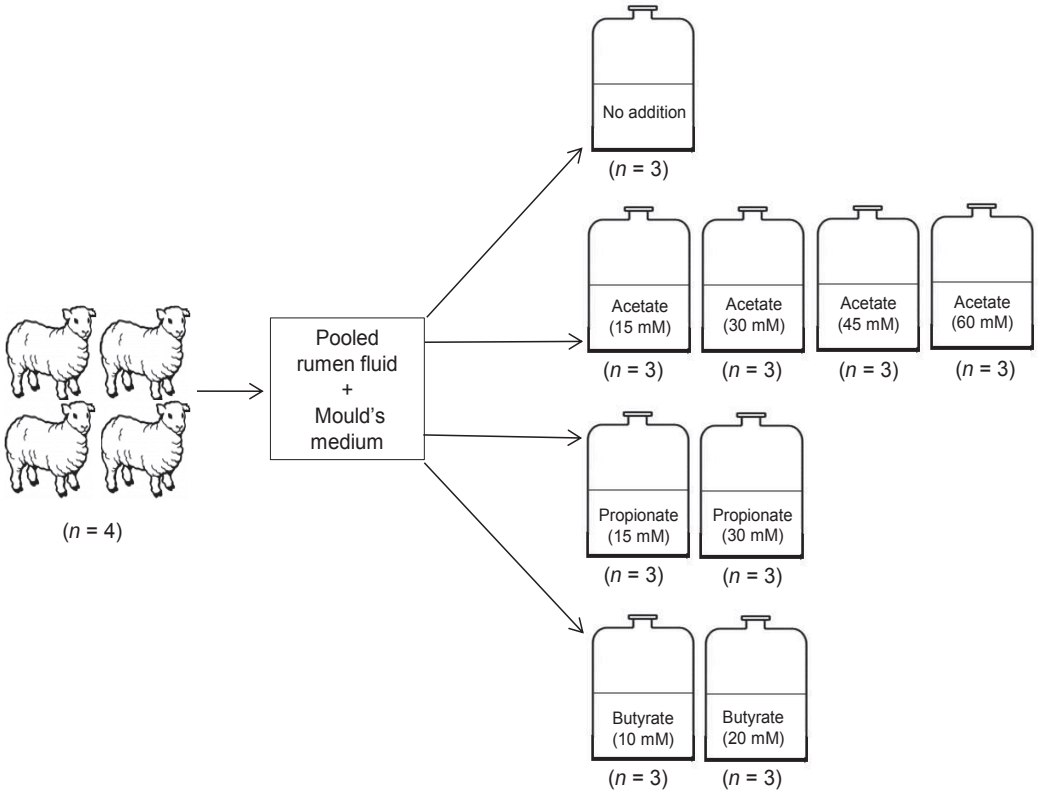

Figure 4.2 Experiment 2. Overview of the short-term *in vitro* experiment used to measure the effects of addition of large amounts of VFA on rumen fermentation. Methane, hydrogen and total gas production were continuously measured using gas chromatography (GC). Liquid samples (2 ml) were collected after 8 hours to measure VFA concentrations.

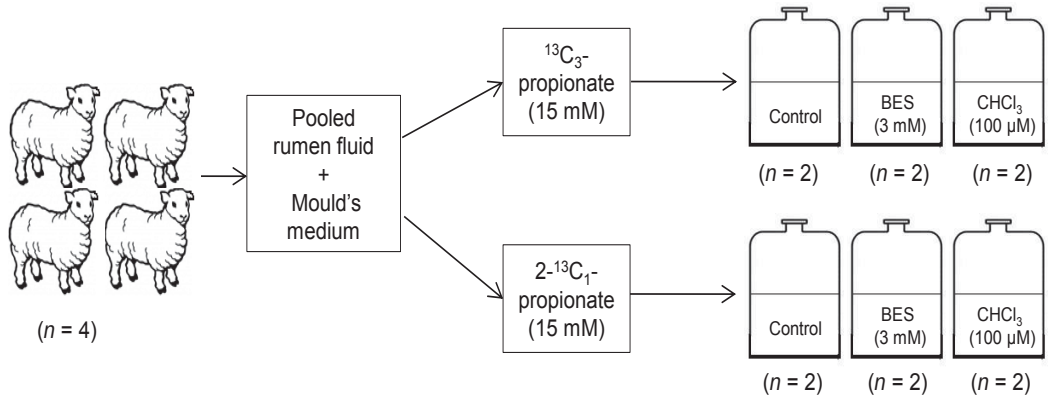

Figure 4.3 Experiment 3. Overview of the short-term *in vitro* experiment used to measure VFA inter-conversion from propionate to acetate and butyrate. Liquid samples (2 ml) were collected every two hours to measure VFA concentrations and the fractional amounts of ^{13}C-labelled propionate converted to acetate and butyrate.

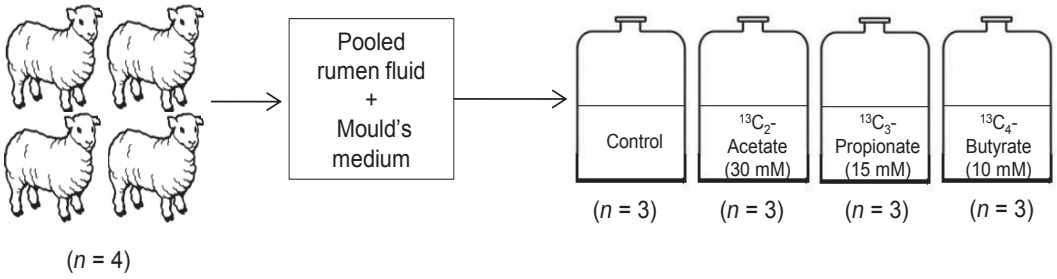

Figure 4.4 Experiment 4. Overview of the short-term *in vitro* experiment used to measure VFA inter-conversion between acetate, propionate and butyrate. Liquid samples (2 ml) were collected every two hours to measure VFA concentrations and the fractional amounts of inter-conversion of acetate, propionate and butyrate. This experiment was repeated twice on two separate days.

The four experiments outlined in Figures 4.1, 4.2, 4.3 and 4.4 were carried on four separate days. Briefly, in Experiment 1, 200 ml of rumen fluid were collected from each of four fistulated, pasture-fed sheep, whereas in Experiments 2, 3 and 4, 200 ml of rumen fluid was collected from the four fistulated sheep, housed in pens and adapted to general purpose (GP) diet for a period of two weeks. The rumen fluid was filtered through 1 mm mesh-size cheese cloth (Stockinette, Cirtex Industries, Thames, New Zealand), and the filtrates were pooled together and diluted five-fold (v/v) with Mould's medium (pH 6.5; temperature 39 °C, under 100% CO_2 atmosphere; Mould *et al.* 2005). Sixty millilitres of this diluted sample were added to 120 ml volume serum bottles held at 39 °C, each of which contained

0.6 g of dried ryegrass (Experiment 1) or GP diet (Experiment 2, 3 and 4). Inhibitors and tracer substrates were added as required.

Experiment 1. Inhibitors (10 mM BES or 2 mM chloroform) or an equivalent volume of Mould's medium (the actual assay with no inhibitor) were added as required. One set of three bottles was then supplemented with 3 mM $NaH^{13}CO_3$, whereas a second set of three bottles did not receive $NaH^{13}CO_3$ (Figure 4.1).

Experiment 2. Bottles were supplemented with 15, 30, 45 and 60 mM acetate, 15 and 30 mM propionate, 10 and 20 mM butyrate; or an equivalent volume of only Mould's medium as a control (Figure 4.2).

Experiment 3. Inhibitors (3 mM BES or 100 µM chloroform) or an equivalent volume of Mould's medium (control) were added as required. One set of three bottles was supplemented with 15 mM $^{13}C_3$-propionate, whereas a second set of three bottles received 15 mM 2-$^{13}C_1$-propionate (Figure 4.3).

Experiment 4. Bottles were supplemented with 30 mM $^{13}C_2$-acetate, 15 mM $^{13}C_3$-propionate, 10 mM $^{13}C_4$-butyrate, or an equivalent volume Mould's medium (control; Figure 4.4).

The bottles were sealed with rubber stoppers and incubated for 8 h at 39 °C in a shaking incubator coupled to a GC system that continuously measured methane, hydrogen and total gas production (Muetzel *et al.* 2014). Incubations were carried out in triplicate in Experiment 1, 2 and 4 and duplicate in Experiment 3, and samples collected as outlined in Figures 4.1, 4.2, 4.3 and 4.4. Experiment 4 was repeated twice on two separate days. Liquid fermentation samples were collected every two hours and analysed for VFA by gas chromatography-flame ionisation detection (GC-FID) and high-performance liquid chromatography (HPLC). The ratio of $^{13}C/^{12}C$ in dissolved (CO_2) and headspace gas (CO_2 and CH_4) samples was measured by gas chromatography-isotope ratio mass spectrometry (GC-IRMS). Homoacetogenesis was measured by measuring the incorporation of $^{13}CO_2$ into ^{13}C-acetate after 8 hours of incubation by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) using solid-phase micro extraction (SPME). Labelled VFA were measured using gas chromatography-mass spectrometry (GC-MS). A

more detailed description of the experimental set-up and methods used can be found in Chapter 2.

Statistical analysis of data

One way ANOVA was used to compare treatment followed by Dunnett's multiple comparison with control using the Minitab software analysis tool (version 17; Minitab Inc., Pennsylvania, USA). The significance of all effects were set at 5% ($p < 0.05$), and the effects with p-values more than 5% and less than 10% were regarded as a trend towards an increase or decrease ($p = 0.05$ to 0.10).

4.4 Results and discussion

4.4.1 Experiment 1. Development of an *in vitro* assay to measure homoacetogenesis

The assay measured the incorporation of $^{13}CO_2$ into acetate as a measure of homoacetogenesis. Each assay consisted of three different treatments: the assay itself in which the homoacetogenic activity in the inoculum was measured, and two additional treatments where an inhibitor of methanogenesis (BES), and of both methanogenesis and homoacetogenesis (chloroform) was added. The expected effects of the treatments are given in Table 4.1. This short-term *in vitro* assay represents the activity in the rumen at the time of sampling, and can be used to estimate VFA formation, which is confounded in the animal by uptake and passage from the rumen.

Table 4.1 Expected effects of inhibitors *in vitro* (mixed culture experiment).

Inhibitors	Effects
None	Normal methanogenesis and homoacetogenesis
BES (10 mM)	Inhibit methanogenesis and increase homoacetogenesis
Chloroform (2 mM)	Inhibit methanogenesis and inhibit or decrease homoacetogenesis

4.4.1.1 Methane production was inhibited by addition of inhibitors

In the assays without any added inhibitor, a very small amount of hydrogen (< 0.29% v/v of total gas) and a large amount of methane was produced. The amount of methane increased over time and was approximately 0.37 mmol/bottle (12.6% v/v of total gas) at 8 h (Figure 4.5a). In contrast, in the assays with added BES, there was a 95.8% reduction in methane ($p < 0.0001$) as compared to the assays without inhibitor, and the amount of hydrogen formed increased significantly by 30.8-fold ($p < 0.0001$) and reached approximately 0.27 mmol/bottle (8.55% v/v of total gas) at 8 h (Figure 4.5b) compared to the assays without inhibitor. With the addition of chloroform, the amount of methane formed was reduced significantly ($p < 0.0001$) by 98.2% compared to the assays without inhibitor, and the amount of hydrogen formed increased significantly by 31.9-fold ($p < 0.0001$) and was approximately 0.28 mmol/bottle (8.78% v/v of total gas) at 8 h (Figure 4.5c) compared to the assays without inhibitor. Total gas was comprised mainly of CO_2, and total gas production was not affected by the addition of inhibitors (BES; $p = 0.500$ or chloroform; $p = 0.505$) as compared to the assays without inhibitor. There was no difference in gas production between bottles that received $NaH^{13}CO_3$ or that did not receive $NaH^{13}CO_3$ (compare Figure 4.5a and 4.5d). This implies that the addition of inhibitors and $NaH^{13}CO_3$ did not affect the extent of feed degradation *in vitro*. The inhibitors did, however, have a marked effect on methane formation, presumably from the hydrogen generated from the feed. However, the amount of hydrogen formed in the presence of the inhibitors (0.27 or 0.28 mmol/bottle) was only 18.6% to 19.3% of that expected from the reduction in the amount of methane, assuming 4 mol of H_2 is converted to 1 mol of CH_4.

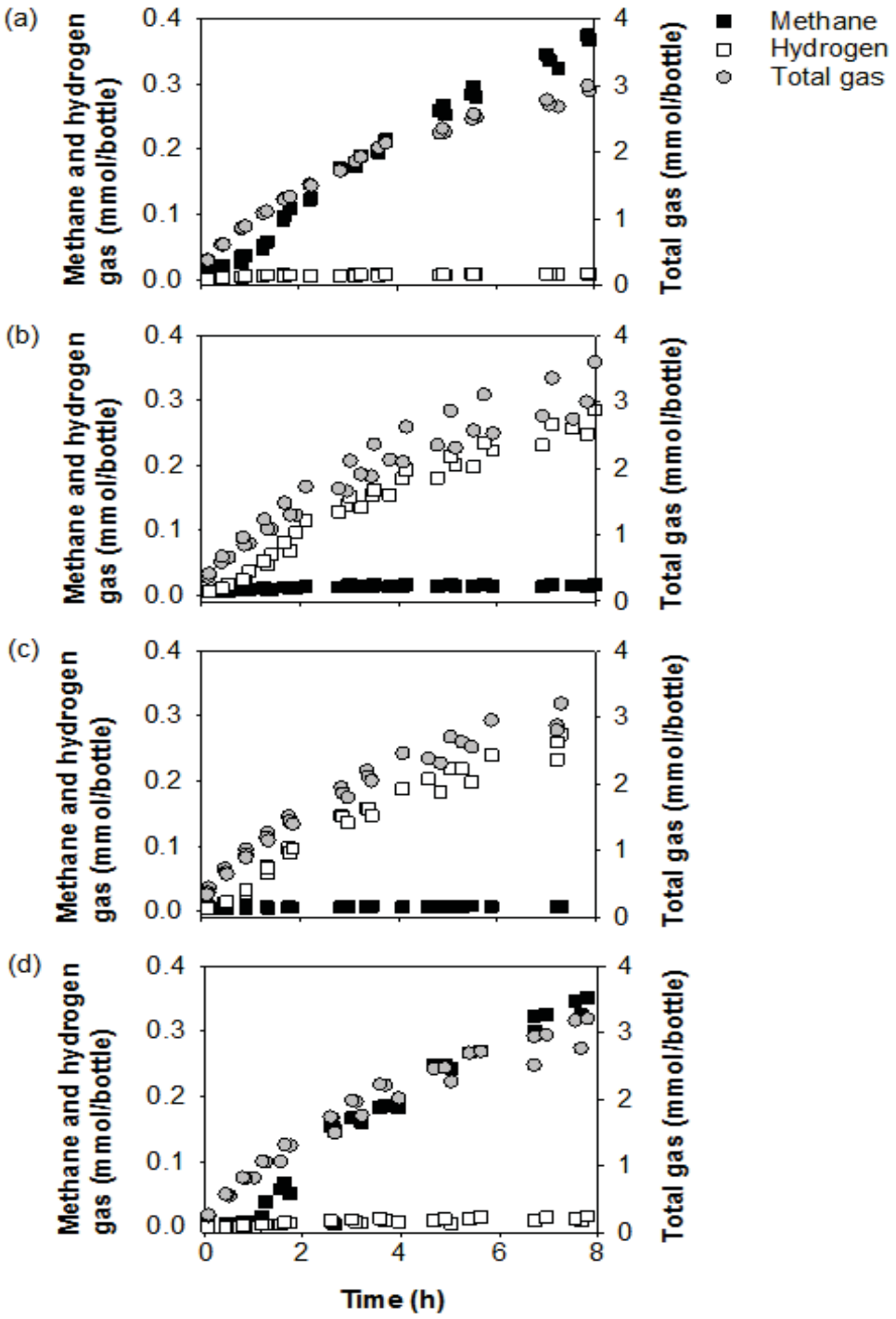

Figure 4.5 Methane, hydrogen and total gas produced by sheep rumen contents incubated *in vitro* in the absence of inhibitor (a) and in the presence of BES (b) or chloroform (c) with $NaH^{13}CO_3$ not added, and in the absence of inhibitor with $NaH^{13}CO_3$ added (d), over time. Data points from all experimental replicates (n = 3) of each treatment are plotted.

4.4.1.2 Shift in volatile fatty acids with inhibition of methane production

There was no significant change in the the quantity of acetate produced ($p = 0.632$), in the presence of the inhibitor BES, but the amount of propionate ($p = 0.010$) and butyrate ($p = 0.001$) formed did increase significantly by 30.4% and 41.9% respectively (Figure 4.6b) as compared to the assays with no inhibitor at 8 h (Figure 4.6a). The addition of chloroform resulted in a significant decrease in acetate ($p = 0.001$) formation by 31.4%, no significant effect in propionate ($p = 0.127$), and a trend towards increase in butyrate ($p = 0.070$) formation by 16.3% in comparison to the assays with no inhibitor at 8 h (Figure 4.6c). The total amount of VFA produced was not significantly affected by addition of BES ($p = 0.181$) or chloroform ($p = 0.102$) as compared the assays with no inhibitor. The VFA profile followed similar pattern in bottles that received $NaH^{13}CO_3$ or did not receive $NaH^{13}CO_3$ (compare Figure 4.6a and 4.6d).

Small amounts of the branched and minor short chain fatty acids isobutyrate, isovalerate, valerate and caproate were detected in each treatment (Figure 4.7). The amount of valerate increased significantly with the addition of BES ($p = 0.001$; Figure 4.7b) and chloroform ($p = 0.007$; Figure 4.7c) by 28.2% and 18.1% respectively as compared to the assays with no inhibitor at 8 h (Figure 4.7a). Isobutyrate ($p = 0.009$) and isovalerate ($p = 0.004$) increased significantly in the presence of BES by 35.5% and 50.4% respectively, while with addition of chloroform isobutyrate ($p = 0.318$) and isovalerate ($p = 0.977$) were not affected, compared to the assays without inhibitor at 8 h. Caproate was detected only at low concentrations in each of the treatments (< 0.02 mmol/bottle).

Lactate, formate and ethanol concentrations were measured, but these were produced in very small amounts, i.e., less than 0.04 mmol/bottle and sometimes even less than 0.01 mmol/bottle (Table 4.2). Therefore, these were not considered further for any calculations.

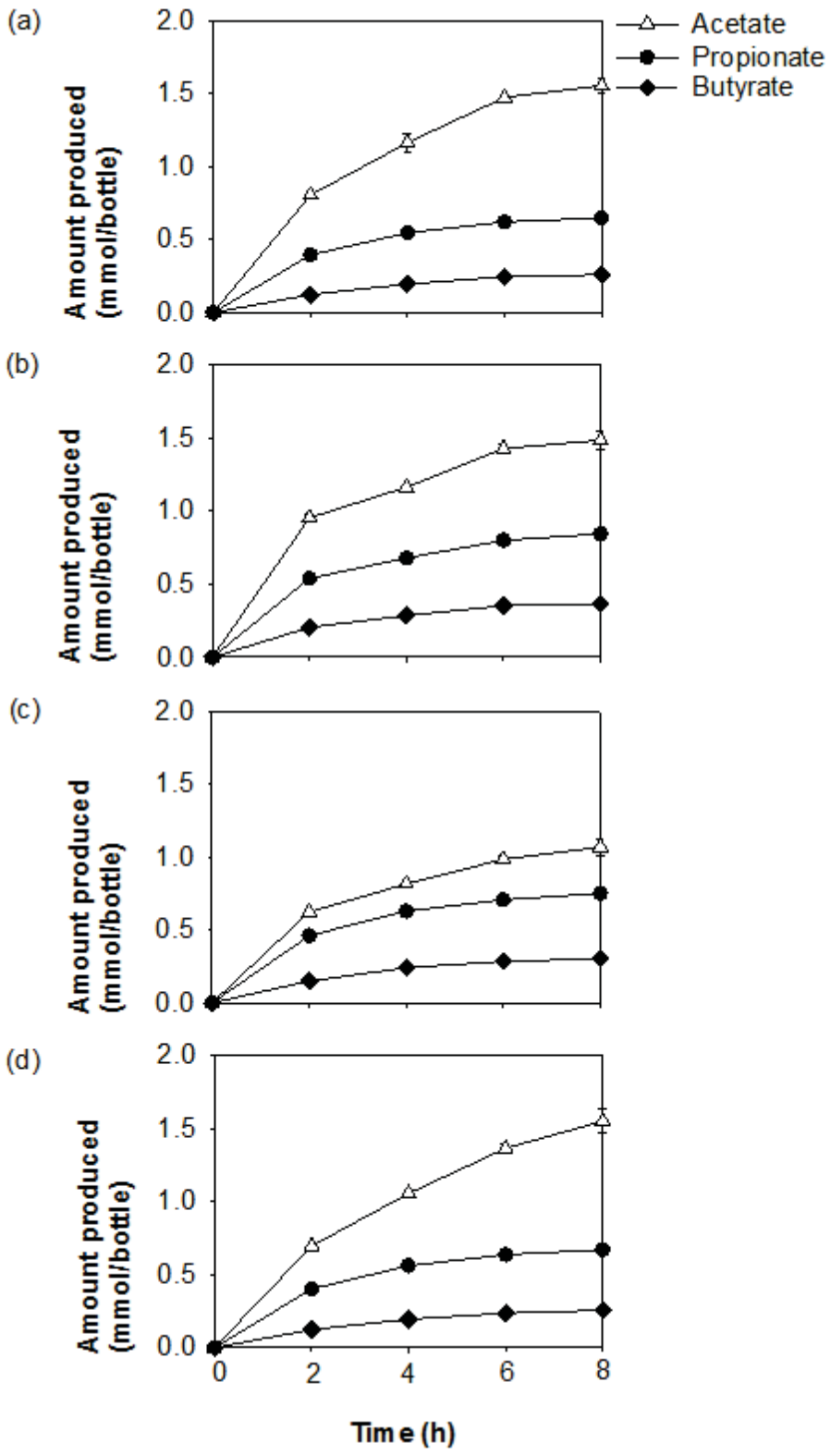

Figure 4.6 Volatile fatty acids (acetate, propionate and butyrate) produced by sheep rumen contents incubated *in vitro* in the absence of inhibitor (a) and in the presence of BES (b) or chloroform (c) with $NaH^{13}CO_3$ not added, and in the absence of inhibitor with $NaH^{13}CO_3$ added (d), over time. Data are presented as means ± standard errors of the mean (n = 3).

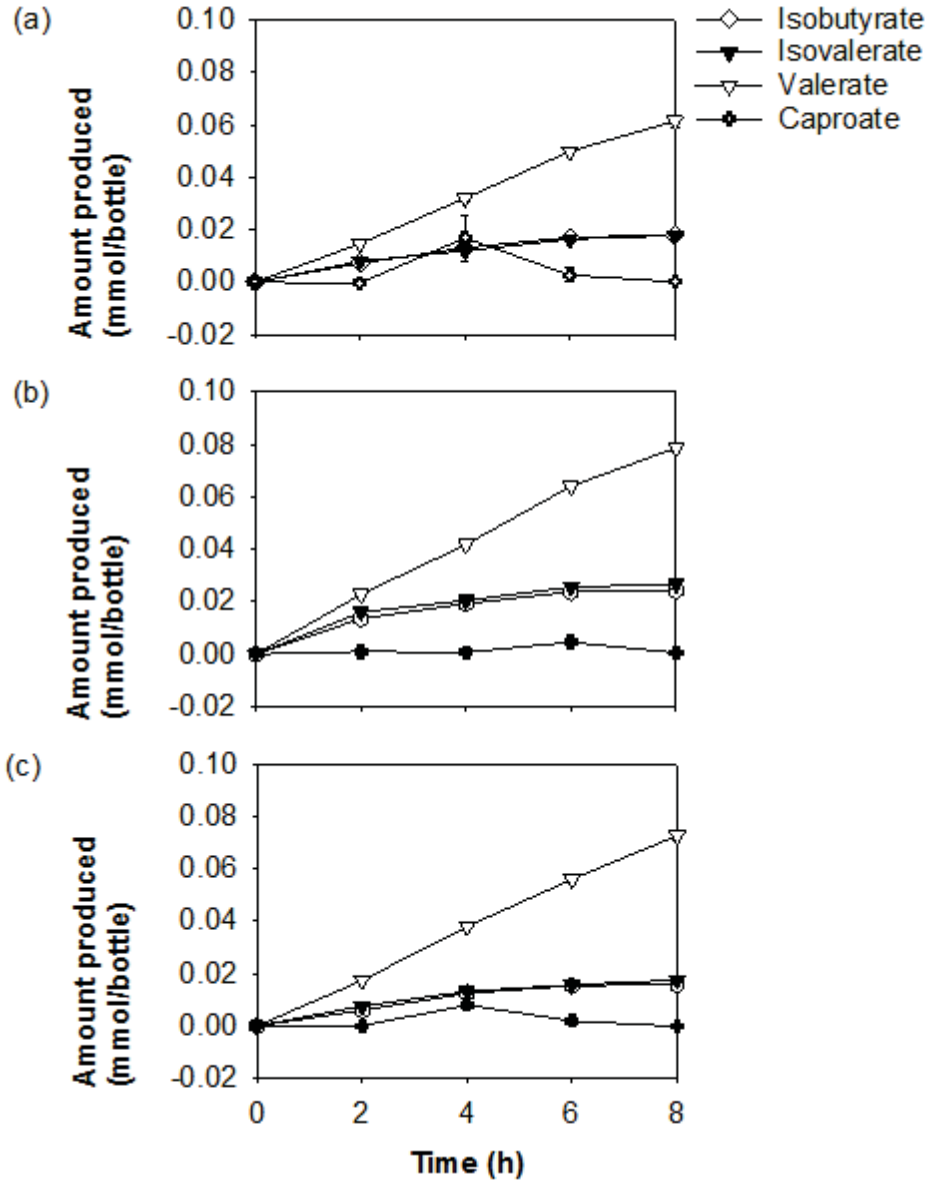

Figure 4.7 Branched and minor short-chain fatty acids (isobutyrate, isovalerate, valerate and caproate) produced in sheep rumen contents incubated *in vitro* in the absence of inhibitor (a) and in the presence of BES (b) or chloroform (c) over time. Data are presented as means ± standard errors of the mean (n = 3).

Table 4.2 Mean amounts of lactate, formate and ethanol produced in the absence of inhibitor and in the presence of BES or chloroform over time.

Inhibitors and products in mmol/bottle (± SEM[a]; *n* = 3[b])									
	No inhibitor			BES			$CHCl_3$		
Time (h)	**Lactate**	**Formate**	**Ethanol**	**Lactate**	**Formate**	**Ethanol**	**Lactate**	**Formate**	**Ethanol**
2	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
4	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
6	< 0.01	< 0.01	0.022 (± 0.011)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
8	< 0.01	< 0.01	< 0.01	< 0.01	0.025 (± 0.0028)	< 0.01	< 0.01	0.036 (± 0.0013)	< 0.01

[a]Standard error of the mean.

[b]No. of experimental replicates.

4.4.1.3 Equilibrium between dissolved and headspace CO_2 and CH_4 was reached within 8 h

The ratio of $^{13}C/^{12}C$ in dissolved CO_2 and headspace CO_2 and CH_4 was analysed every two hours, to determine the equilibrium between the dissolved and gaseous phases, after addition of 3 mM $NaH^{13}CO_3$ (Figure 4.8). As a significant amount of methane gas was produced only in the absence of inhibitor, headspace gas samples were only analysed from these bottles. The ratio of $^{13}C/^{12}C$ in CO_2 in dissolved and headspace gas samples from all the treatments (no inhibitor, BES or chloroform) was approximately 2.5-fold higher than ratio of $^{13}C/^{12}C$ in CO_2 in dissolved and headspace gas samples with no $NaH^{13}CO_3$ added (natural abundance). The ratio of $^{13}C/^{12}C$ in CO_2 in dissolved and headspace gas samples from all the treatments (i.e., no inhibitor, BES or chloroform) came into slow equilibrium with each other over 6 to 8 h. At all times, the enrichment was greater than natural abundance, showing that the $^{13}CO_2$ was not lost over the period of the assay, or diluted to close to natural abundance by the unlabelled CO_2 formed through the fermentation of the feed. This indicated that there was a slow exchange between dissolved and gaseous (headspace) phase CO_2, and that the dissolved $^{13}CO_2$ concentration should be used for calculations of label incorporation. The ratio of $^{13}C/^{12}C$ in CH_4 in the gas phase was 0.0276, as compared to ratio of $^{13}C/^{12}C$ in dissolved (0.0295) and gaseous (0.0292) CO_2 (no inhibitor) at 8 h. Therefore, the ratio of $^{13}C/^{12}C$ of in CH_4 gas phase was 5 to 6% lower than the ratio of $^{13}C/^{12}C$ in dissolved and gaseous CO_2 samples (no inhibitor). This seems to be consistent with the reported level of fractionation occurring during hydrogenotrophic methanogenesis, i.e., 5% less ^{13}C-content in methane relative to CO_2 (Krzycki *et al.* 1987).

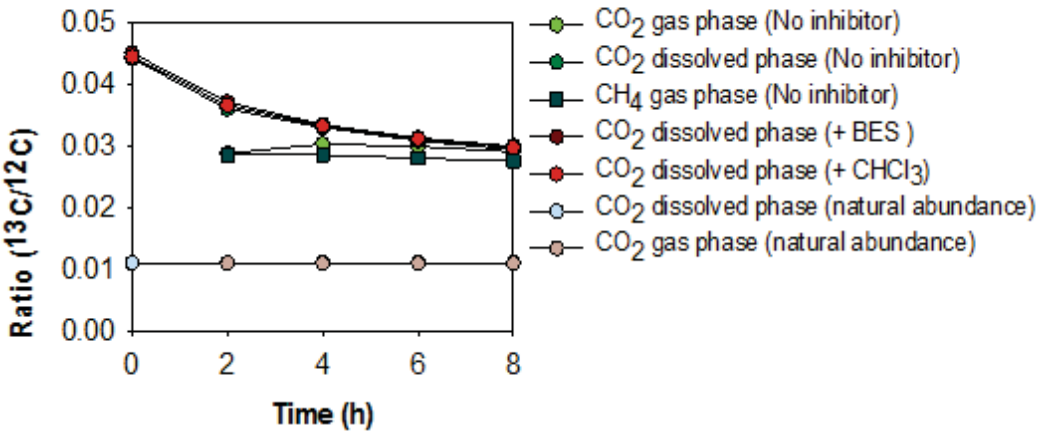

Figure 4.8 Ratios of $^{13}CO_2$ to $^{12}CO_2$ or $^{13}CH_4$ to $^{12}CH_4$ in the liquid phase or in the headspace when sheep rumen contents were incubated *in vitro* in the absence of inhibitor and presence of BES or chloroform over time. Data are presented as means ± standard errors of the mean (n = 3).

4.4.1.4 Formation of ^{13}C-labelled acetate, propionate and butyrate from $^{13}CO_2$

The incorporation of $^{13}CO_2$ into ^{13}C-acetate, ^{13}C-propionate and ^{13}C-butyrate was measured using GC-IRMS (Chapter 2; Section 2.18.5). The ratio of $^{13}C/^{12}C$ was determined for acetate, propionate and butyrate after 8 h of *in vitro* incubation in the assays without inhibitor and in the presence of BES or chloroform. The enrichment data from IRMS combined with the ruminal VFA data (GC-FID) were used to calculate the amount of excess labelled acetate (^{13}A), propionate (^{13}P) and butyrate (^{13}B) present after 8 h, using equations (1), (2) and (3) respectively.

$$^{13}A = (r\mathrm{A}_{\mathrm{Lferm}} - r\mathrm{A}_{\mathrm{Uferm}})/(r\mathrm{A}_{\mathrm{Lferm}} + 1) \times A \quad (1)$$

$$^{13}P = (r\mathrm{P}_{\mathrm{Lferm}} - r\mathrm{P}_{\mathrm{Uferm}})/(r\mathrm{P}_{\mathrm{Lferm}} + 1) \times P \quad (2)$$

$$^{13}B = (r\mathrm{B}_{\mathrm{Lferm}} - r\mathrm{B}_{\mathrm{Uferm}})/(r\mathrm{B}_{\mathrm{Lferm}} + 1) \times B \quad (3)$$

where $r\mathrm{A}_{\mathrm{Lferm}}$ and $r\mathrm{A}_{\mathrm{Uferm}}$ are the ratios of $^{13}C/^{12}C$ in acetate in fermentations with $NaH^{13}CO_3$ and with unlabelled $NaHCO_3$ respectively, $r\mathrm{P}_{\mathrm{Lferm}}$ and $r\mathrm{P}_{\mathrm{Uferm}}$ are the ratios of $^{13}C/^{12}C$ in propionate from labelled and unlabelled fermentations, and $r\mathrm{B}_{\mathrm{Lferm}}$ and $r\mathrm{B}_{\mathrm{Uferm}}$ are the ratios of $^{13}C/^{12}C$ in butyrate from labelled and unlabelled fermentations. A, B and P are the amounts of acetate, butyrate and propionate produced after 8 h (measured using GC-FID) respectively.

The amount of ^{13}A produced was 0.31 µmol/bottle in the assays with no inhibitor. The addition of BES increased it by 41.9% to 0.44 µmol/bottle and chloroform reduced it by 38.7% to 0.19 µmol/bottle as compared to assays with no

inhibitor (Table 4.3). A large amount of ^{13}P was also observed, around 3.36 µmol/bottle in the assays with no inhibitor, and this increased with the addition of BES by 20.5% to 4.05 µmol/bottle and with the addition of chloroform by 20.8% to 4.06 µmol/bottle. Labelled butyrate (^{13}B), which was 0.046 µmol/bottle in the assays with no inhibitor, also increased with addition of BES by 89.1% to 0.087 µmol/bottle and with chloroform it increased by 23.9% to 0.057 µmol/bottle, as compared to assays with no inhibitor. Therefore, apart from acetate (^{13}A), a large amount of ^{13}C-label also appeared in propionate (^{13}P) and only very small amounts in butyrate (^{13}B).

4.4.1.5 Homoacetogenic activity in fresh rumen fluid estimated using short-term assay

To determine whether homoacetogenesis occurred in sheep rumen fluid and if it could be measured, the incorporation of $^{13}CO_2$ into ^{13}C-acetate after 8 h of *in vitro* incubation was measured. The time (8 h) for sampling to measure homoacetogenesis was chosen to allow sufficient time for labelling to occur. Gas and VFA formation started to slow after 8 h, meaning that longer incubations would not give a valid estimation of incorporation rates. The enrichment data from GC-IRMS (Table 4.3) were used to calculate the fraction of acetate (X_a) coming from CO_2 as:

$$X_a = (rA_{Lferm} - rA_{Uferm})/(rCO_{2\ Lferm} - rA_{Uferm}) \quad (4)$$

where $rCO_{2\ Lferm}$ is the ratio of $^{13}C/^{12}C$ in CO_2 for the labelled fermentation.

The amount of acetate produced from homoacetogenesis (A_{ha}') was calculated by incorporating the value of X_a from equation (4) and the total acetate produced (A), determined by GC-FID, into equation (5).

$$A_{ha}' = X_a \times A \quad (5)$$

Acetate formed from homoacetogenesis was expressed as both the amount of acetate produced from homoacetogenesis in µmol/bottle (Figure 4.9a) and amount of acetate from homoacetogenesis as a percentage of total acetate produced ($X_a \times 100$; Figure 4.9b).

Table 4.3 Mean of ratios of $^{13}C/^{12}C$ and amounts of ^{13}A, ^{13}P and ^{13}B produced after 8 h *in vitro* incubation.

Mean (± SEM[a]) after 8 h of incubation (n = 3[b])							
$NaH^{13}CO_3$ not added							
Inhibitors	***r*A$_{Uferm}$**	***r*P$_{Uferm}$**	***r*B$_{Uferm}$**	***r*CO$_{2\ Uferm}$**			
None	0.01086 (± 0.000002)	0.01085 (± 0.000004)	0.01082 (± 0.000003)	0.01111 (± 0.000002)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	***r*A$_{LFerm}$**	***r*P$_{LFerm}$**	***r*B$_{LFerm}$**	***r*CO$_{2\ LFerm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.01107 (± 0.000003)	0.01594 (± 0.000043)	0.01100 (± 0.000007)	0.02948 (± 0.000118)	0.31514 (± 0.01256)	3.36292 (± 0.10712)	0.04674 (± 0.00348)
BES	0.01127 (± 0.000001)	0.01612 (± 0.000033)	0.01110 (± 0.000005)	0.03004 (± 0.000212)	0.44185 (± 0.01647)	4.04742 (± 0.10010)	0.08729 (± 0.00187)
$CHCl_3$	0.01103 (± 0.000001)	0.01592 (± 0.000013)	0.01100 (± 0.000018)	0.02991 (± 0.000192)	0.19086 (± 0.00149)	4.05622 (± 0.06393)	0.05723 (± 0.00722)

[a]Standard error of the mean.

[b]No. of experimental replicates.

The amount of acetate formed from homoacetogenesis in sheep rumen fluid incubated *in vitro* for 8 h was 17.1 µmol/bottle in the assays with no inhibitor (Figure 4.9a). Acetate from homoacetogenesis increased by 36.3% as compared to assays with no inhibitor, to 23.3 µmol/bottle with inhibition of methane production in the presence of BES over 8 h. This confirmed that there was capacity for homoacetogenesis to increase if methane formation was inhibited, presumably because homoacetogens could use the increased hydrogen available in the absence of active methanogens. The amount of acetate from homoacetogenesis was reduced by 40.9% as compared to assays with no inhibitor, to approximately 10.1 µmol/bottle with the addition of chloroform, which inhibits both methanogens and homoacetogens (Chapter 3). The inhibition of homoacetogenesis in the mixed *in vitro* system appears to be incomplete. These results confirmed the occurrence of homoacetogenesis in the presence of methanogenesis (the assays with no inhibitor), showed a potential to increase in the absence of methanogenesis (with the addition of BES), and a decrease when both methanogenesis and homoacetogenesis were inhibited (with chloroform) during short-term *in vitro* incubations (8 h).

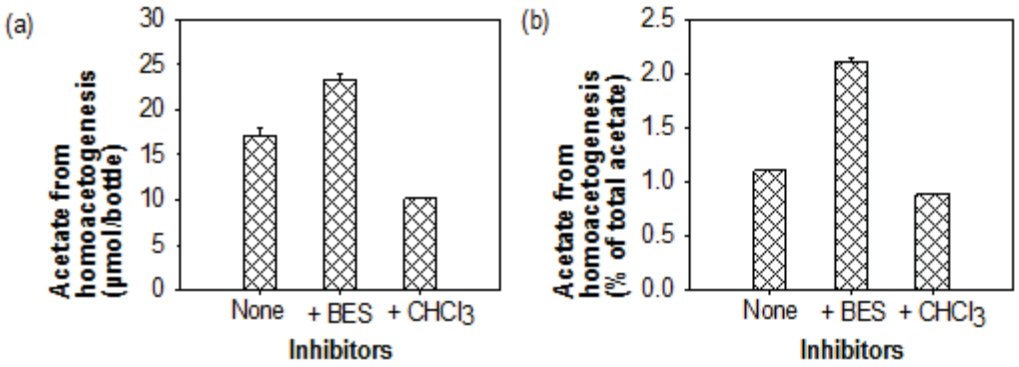

Figure 4.9 Amount of acetate (a) and percentage of total acetate (b) produced from homoacetogenesis following incubation of sheep rumen contents *in vitro* after 8 h in the absence of inhibitor and presence of BES or chloroform over time. Data are presented as means ± standard errors of the mean (n = 3).

4.4.1.6 Accounting for butyrate from homobutyrogenesis

Butyrate can also be formed through the activity of homoacetogens, in what can be termed "homobutyrogenesis" (see Section 1.4.1). The fraction of butyrate formed from homobutyrogenesis (X_b), i.e., coming from CO_2, was calculated from the amount of label incorporated into butyrate during the *in vitro* incubation. This was calculated using enrichment data from GC-IRMS (Table 4.3) as:

$$X_b = (rB_{Lferm} - rB_{Uferm})/(rCO_{2\ Lferm} - rB_{Uferm}) \quad (6)$$

The amount of butyrate produced from homobutyrogenesis (B_{hb}') was calculated by incorporating the value of X_b from equation (6) and the total butyrate produced (B) determined by GC-FID, into equation (7).

$$B_{hb}' = X_b \times B \quad (7)$$

Butyrate formed from homobutyrogenesis was expressed as both the amount of butyrate produced from homobutyrogenesis in µmol/bottle (Figure 4.10a) and amount of butyrate from homobutyrogenesis as a percentage of total butyrate produced ($X_b \times 100$; Figure 4.10b). The amount of butyrate formed from homobutyrogenesis in sheep rumen fluid incubated *in vitro* for 8 h was 2.53 µmol/bottle in the assays with no inhibitor (Figure 4.10a). With the addition of BES, it increased by 81.4% as compared to assays with no inhibitor, to approximately 4.59 µmol/bottle, while addition of chloroform increased it by a smaller amount (20.2%) as compared to assays with no inhibitor, to approximately 3.04 µmol/bottle as compared to the assays with no inhibitor. Therefore, with inhibition of methane production by BES or chloroform, homobutyrogenesis increased, but the increase was smaller in the presence of chloroform, in keeping with it being a partial inhibitor of homoacetogenic activity.

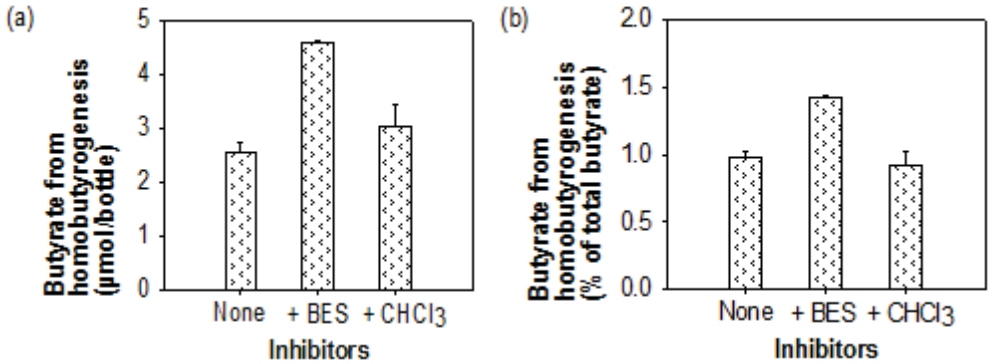

Figure 4.10 Amount of butyrate (a) and percentage of total butyrate (b) produced from homobutyrogenesis following incubation of sheep rumen contents *in vitro* after 8 h in the absence of inhibitor and presence of BES or chloroform over time. Data are presented as means ± standard errors of the mean (n = 3).

4.4.2 Experiment 2. Effect of addition of large concentrations of VFA on *in vitro* rumen fermentation

High concentrations of VFA needed to be added in the next experiments to measure VFA inter-conversions. Therefore, the impacts of the addition of large concentrations of VFA on *in vitro* rumen fermentation was measured to ensure that rumen fermentation was not affected.

4.4.2.1 Gas production was not affected by addition of large concentrations of VFA

Methane, hydrogen and total gas seemed unaffected even when large concentrations of acetate, propionate and butyrate were added to *in vitro* incubation of rumen fluid (Figure 4.11). Even with the addition of 60 mM acetate, the highest concentration of acetate added, the amount of methane produced was 0.43 mmol/bottle ($p = 1.000$), hydrogen 0.013 mmol/bottle ($p = 0.806$) and total gas 3.59 mmol/bottle ($p = 0.999$) (Figure 4.11e) and was comparable to the controls with no addition, i.e., methane 0.43 mmol/bottle, hydrogen 0.011 mmol/bottle and total gas 3.61 mmol/bottle (Figure 4.11a). With the addition of highest concentration of propionate (30 mM), the amount of methane produced was 0.41 mmol/bottle ($p = 1.000$), hydrogen 0.011 mmol/bottle ($p = 1.000$) and total gas 3.44 mmol/bottle ($p = 1.000$) and were comparable to the controls with no addition (Figure 4.11g). Addition of butyrate at a concentration of 20 mM, resulted into formation of 0.39 mmol/bottle methane ($p = 0.995$), 0.012 mmol/bottle hydrogen ($p = 0.991$) and 3.29 mmol/bottle of total gas ($p = 0.998$), which was comparable to control with no addition (Figure 4.11i). This confirmed that addition of large concentrations of VFA into the *in vitro* system barely affected gas production.

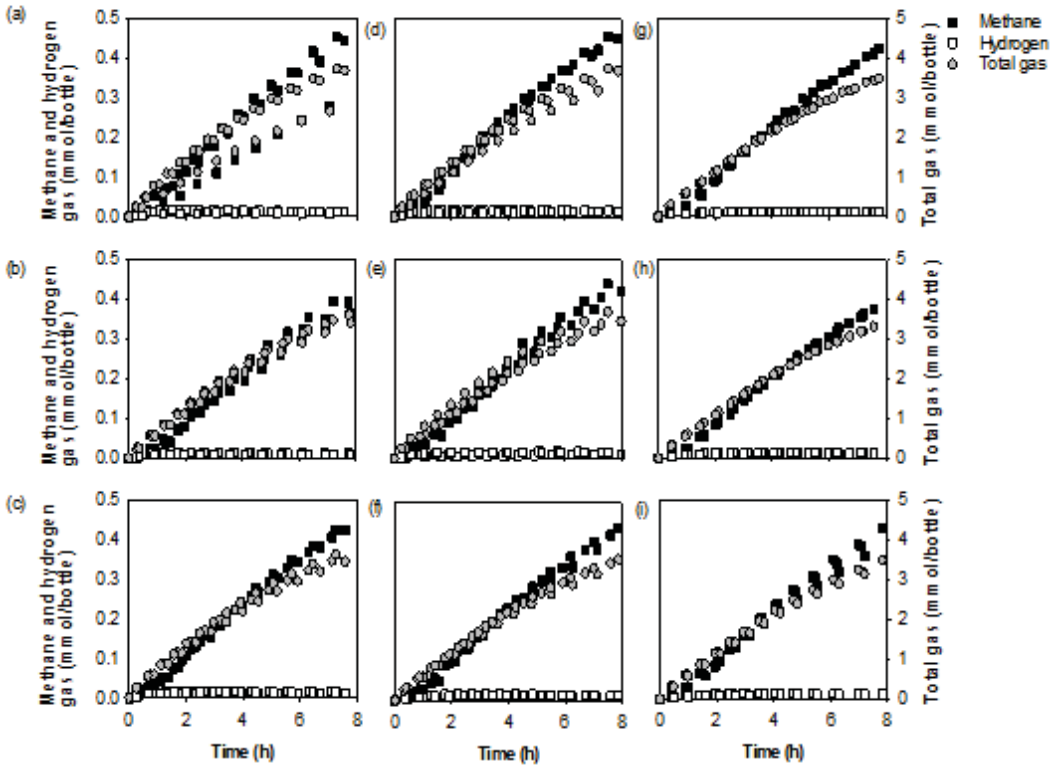

Figure 4.11 Methane, hydrogen and total gas produced by sheep rumen contents incubated *in vitro* over time. Rumen contents were incubated with no addition (a), in the presence of 15 mM (b), 30 mM (c), 45 mM (d) and 60 mM (e) acetate, 15 mM (f) and 30 mM (g) propionate, and 10 mM (h) and 20 mM (i) butyrate. The left and right axes apply to all panels. Data are presented as means ± standard deviation (n = 3).

4.4.2.2 *In vitro* VFA production was slightly affected by addition of large concentrations of VFA

As compared to the control with no addition, the addition of 15 mM ($p = 0.854$), 30 mM ($p = 0.143$) and 45 mM ($p = 0.057$) acetate had no significant effect on the amount of acetate formed after 8 h of *in vitro* incubation of rumen fluid, whereas 60 mM acetate ($p = 0.037$) appeared to significantly effect the amount of acetate, formed, reducing it by 19.6% (Figure 4.12a). Addition of 15 mM ($p = 0.883$), 30 mM ($p = 0.731$) 45 mM (0.852), and 60 mM ($p = 0.998$) acetate had no significant effects on the amounts of propionate. Similarly, 15 mM ($p = 0.281$), 30 mM ($p = 1.000$), 45 mM ($p = 1.000$), and 60 mM ($p = 1.000$) addition of acetate had no significant effects on the amounts of butyrate after 8 h as compared to control with no addition. Addition of 15 mM propionate had no significant effects on the amounts of acetate ($p = 0.984$) or propionate ($p = 0.198$) formed, but there was a trend to less butyrate formation ($p = 0.082$) as compared to control with no additions, although the reduction was only 8.73%. Addition of 30 mM propionate

had no effects on amount of acetate (p =0.982), but propionate (p = 0.001) and butyrate (p = 0.021) were significantly decreased by 27.5% and 10.9% respectively (Figure 4.12b). Addition of 10 mM butyrate had no effects on acetate (p = 0.841) and propionate (p = 0.882), however decreased butyrate formation significantly (p < 0.0001) although the reduction was 25.3%, while addition of 20 mM butyrate had no significant effects on amounts of acetate (p = 0.973) and propionate (p = 0.844) formed, but significantly decreased the amount of butyrate formed (p < 0.0001) and the reduction was 32.2% and greater then as compared with 10 mM butyrate addition(Figure 4.12c). This showed that addition of large concentrations of VFA into *in vitro* system had only small effects on VFA production. Therefore, 30 mM acetate, 15 mM propionate and 10 mM butyrate were considered suitable concentrations for *in vitro* addition because they had no significant imapcts on VFA production.

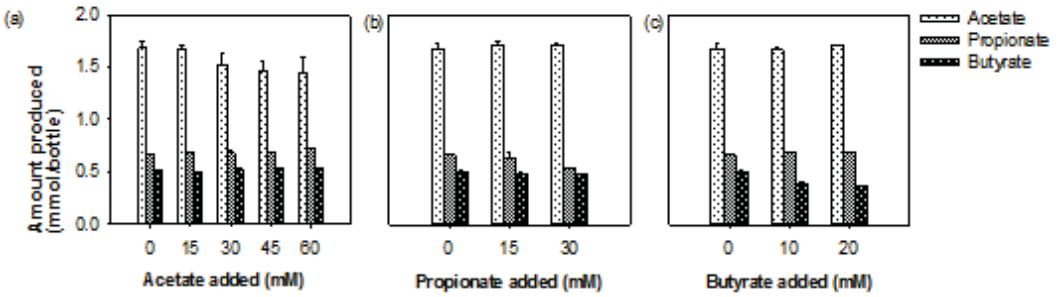

Figure 4.12 Acetate, propionate and butyrate produced by sheep rumen contents incubated *in vitro* after 8 h with the addition of different amounts of acetate (a), propionate (b) and butyrate (c). The left axes apply to all panels. Data are presented as means ± standard errors of the mean (n = 3) and the initial and added VFA were subtracted.

4.4.3 Experiment 3. VFA inter-conversion from propionate to acetate and butyrate

When rumen fluid was incubated with $NaH^{13}CO_3$ in Experiment 1, a large amount of ^{13}C-label was also observed in propionate and this increased with addition of BES (Table 4.3). It was therefore not certain whether labelled acetate or butyrate was directly derived from CO_2 or whether VFA inter-conversion could account for the results observed. Experiment 3 (Figure 4.3 and 4.13) was carried out to measure the amount of ^{13}C-label from propionate transferred to acetate or butyrate via VFA inter-conversion. Rumen contents were incubated in the absence and presence of

inhibitors (BES or chloroform) and tracer substrates ($^{13}C_3$-propionate or 2-$^{13}C_1$-propionate). The amounts of ^{13}C-labelled VFA (acetate and butyrate) formed due to VFA inter-conversion from $^{13}C_3$-propionate or 2-$^{13}C_1$-propionate was measured using GC-MS (Chapter 2; Section 2.18.6) and are summarised in Table 4.4.

The amount of $^{13}C_1$-acetate formed from $^{13}C_3$-propionate in the control incubations with no inhibitor was 3.05 µmol/bottle, whereas much more $^{13}C_2$-acetate was formed, at 23.7 µmol/bottle. The amount of $^{13}C_1$-butyrate formed was 0.38 µmol/bottle, $^{13}C_2$-butyrate 0.85 µmol/bottle, $^{13}C_3$-butyrate 0.33 mol/bottle and $^{13}C_4$-butyrate 0.11 µmol/bottle. Addition of BES or chloroform reduced the amount of ^{13}C-labelled acetate formed from $^{13}C_3$-propionate by 73.1% to 80.8% or butyrate formed by as much as 22.2%. With addition of 2-$^{13}C_1$-propionate, the amount of $^{13}C_1$-acetate formed was 21.4 µmol/bottle and considerably greater than $^{13}C_2$-acetate by 99.1%, which was only 0.19 µmol/bottle. The amount of labelled butyrate formed was very small and only $^{13}C_1$-butyrate was formed in any notable amount, 0.57 µmol/bottle. The amount of $^{13}C_1$-acetate formed from 2-$^{13}C_1$-propionate was reduced by 45.2-52.2% with addition of BES or chloroform, whereas $^{13}C_1$-butyrate formation increased with addition of BES or chloroform by 23.6-23.9%.

The data summarised in Table 4.4 were then used to calculate the fractional amounts of VFA inter-conversion. The fractional amounts of $^{13}C_3$-propionate converted to $^{13}C_2$-acetate (f_{pa}) and $^{13}C_3$-butyrate (f_{pb}) were calculated according to equations (8) and (9) respectively. As the amounts of $^{13}C_1$-acetate, $^{13}C_1$-butyrate and $^{13}C_4$-butyrate formed were much smaller, these were not included in these calculations.

$$f_{pa} = A^{**}/P^{***} \quad (8)$$

$$f_{pb} = B^{***}/P^{***} \quad (9)$$

where A^{**} is the amount of $^{13}C_2$-acetate formed, B^{***} is the amount of $^{13}C_3$-butyrate formed and P^{***} is the amount of $^{13}C_3$-propionate added (900 µmol/bottle).

The fractional amount of 2-$^{13}C_1$-propionate converting to $^{13}C_1$-acetate (f_{pa}) and $^{13}C_1$-butyrate (f_{pb}) was also calculated according to equations (10) and (11) respectively.

Table 4.4 Mean amounts of ^{13}C-labelled acetate and butyrate formed from propionate due to VFA inter-conversion after 8 h *in vitro* incubation in the absence of inhibitor (control) and in the presence of BES or chloroform.

Mean (± SEM[a]) amounts of ^{13}C-labelled VFA formed (µmol/bottle) after 8 h of incubation (n = 2[b])						
$^{13}C_3$-propionate (15 mM added; 900 µmol/bottle)						
Inhibitors	$^{13}C_1$-acetate (A^*)	$^{13}C_2$-acetate (A^{**})	$^{13}C_1$-butyrate (B^*)	$^{13}C_2$-butyrate (B^{**})	$^{13}C_3$-butyrate (B^{***})	$^{13}C_4$-butyrate (B^{****})
Control	3.05 (± 0.234)	23.66 (± 0.326)	0.38 (± 0.164)	0.85 (± 0.123)	0.33 (± 0.010)	0.11 (± 0.006)
BES	0.59 (± 0.286)	12.00 (± 0.217)	0.30 (± 0.043)	0.83 (± 0.099)	0.27 (± 0.011)	0.11 (± 0.046)
$CHCl_3$	0.82 (± 0.168)	11.19 (± 0.427)	0.50 (± 0.017)	0.84 (± 0.037)	0.26 (± 0.005)	0.11 (± 0.021)
2-$^{13}C_1$-propionate (15 mM added; 900 µmol/bottle)						
Inhibitors	$^{13}C_1$-acetate (A^*)	$^{13}C_2$-acetate (A^{**})	$^{13}C_1$-butyrate (B^*)	$^{13}C_2$-butyrate (B^{**})	$^{13}C_3$-butyrate (B^{***})	$^{13}C_4$-butyrate (B^{****})
Control	21.39 (± 0.790)	0.19 (± 0.097)	0.57 (± 0.369)	–0.01 (± 0.008)	0.03 (± 0.014)	0.02 (± 0.002)
BES	11.71 (± 0.450)	0.29 (± 0.019)	0.70 (± 0.222)	0.21 (± 0.062)	–0.05 (± 0.005)	0.02 (± 0.008)
$CHCl_3$	10.22 (± 0.451)	0.18 (± 0.127)	0.73 (± 0.089)	–0.02 (± 0.041)	0.07 (± 0.015)	–0.03 (± 0.003)

[a]Standard error of the mean.

[b]No. of experimental replicates.

The amount of $^{13}C_2$-acetate, $^{13}C_2$-butyrate, $^{13}C_3$-butyrate and $^{13}C_4$-butyrate formed were very small, as described above, so these were not used in these calculations.

$$f_{pa} = A^*/P^* \quad (10)$$

$$f_{pb} = B^*/P^* \quad (11)$$

where A^* is the amount of $^{13}C_1$-acetate formed, B^* is the amount of $^{13}C_1$-butyrate formed and P^* is the amount of 2-$^{13}C_1$-propionate added (900 µmol/bottle).

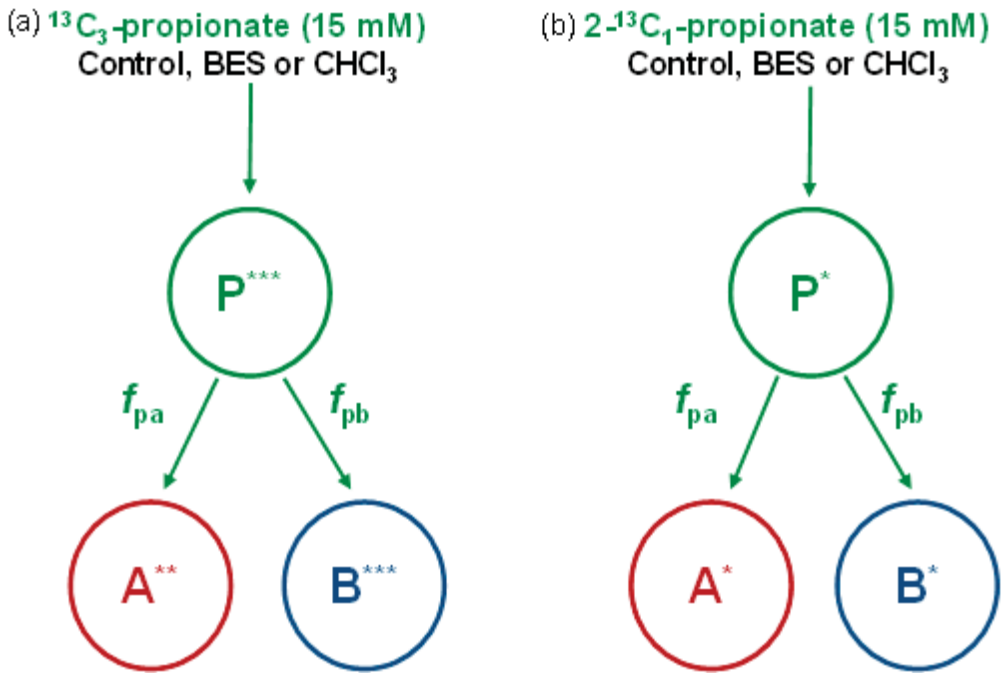

Figure 4.13 One tracer substrate pathway models ($^{13}C_3$-propionate (a) or 2-$^{13}C_1$-propionate (b)) depicting label transfer during 8 h *in vitro* incubation of rumen fluid. The models describe the fractional amounts of labelled propionate converted to labelled acetate (f_{pa}) and butyrate (f_{pb}). In figure (a), A^{**} is the $^{13}C_2$-acetate formed and B^{***} is the $^{13}C_3$-butyrate formed from P^{***}, which is the amount of $^{13}C_3$-propionate added (900 µmol/bottle). In figure (b), A^* is the $^{13}C_1$-acetate formed and B^* is the $^{13}C_1$-butyrate formed from P^*, which is the amount of 2-$^{13}C_1$-propionate added (900 µmol/bottle).

The fractional amounts of conversion of $^{13}C_3$-propionate and 2-$^{13}C_1$-propionate to acetate (f_{pa}) were approximately 0.025 in the control with no inhibitor (Table 4.5). With the addition of BES or chloroform, it was reduced to around 0.013 and 0.011 respectively, i.e., decreased by approximately 48% and 56% as compared to the control with no inhibitor. The fractional amounts of conversion of $^{13}C_3$-propionate and 2-$^{13}C_1$-propionate to butyrate (f_{pb}) in the control with no inhibitor

and in the presence of BES or chloroform were very small (fractional amounts < 0.001).

These results suggests that, in response to addition of BES or chloroform, there was a decreased quantity of label transferring to acetate and butyrate from propionate via VFA inter-conversion relative to what was occurring in the control with no inhibitor.

4.4.4 Experiment 4. VFA inter-conversion between three VFA (acetate, propionate and butyrate)

Rumen contents were incubated in the presence of tracer substrates ($^{13}C_2$-acetate, $^{13}C_3$-propionate, or $^{13}C_4$-butyrate) to determine the fractional amounts of VFA inter-conversion between the three major VFA (acetate, propionate and butyrate). This was to confirm and extend the results from Experiment 3. The amounts of ^{13}C-labelled VFA (acetate, propionate and butyrate) formed due to VFA inter-conversion from $^{13}C_2$-acetate, $^{13}C_3$-propionate or $^{13}C_4$-butyrate were measured using GC-MS (Chapter 2; Section 2.18.6) and are summarised in Table 4.6.

The amount of $^{13}C_2$-propionate formed from $^{13}C_2$-acetate was 37.8 µmol/bottle, while the amounts of $^{13}C_1$-propionate (2.13 µmol/bottle) and $^{13}C_3$-propionate (0.72 µmol/bottle) formed were relatively small. The amounts of $^{13}C_2$-butyrate (81.4 µmol/bottle) and $^{13}C_4$-butyrate (37.7 µmol/bottle) formed from $^{13}C_2$-acetate were much higher than the amounts of $^{13}C_1$-butyrate (2.16 µmol/bottle) and $^{13}C_3$-butyrate (2.80 µmol/bottle). With addition of $^{13}C_3$-propionate, the amount of $^{13}C_1$-acetate was 1.59 µmol/bottle and was less than one-tenth that of $^{13}C_2$-acetate, which was 17.3 µmol/bottle. The amount of labelled butyrate formed was very small and only $^{13}C_2$-butyrate was formed in an appreciable amounts, at 1.40 µmol/bottle. The amount of $^{13}C_1$-acetate formed was 2.69 µmol/bottle and $^{13}C_2$-acetate was 58.6 µmol/bottle with addition of $^{13}C_4$-butyrate, whereas the amount of $^{13}C_2$-propionate formed was 1.00 µmol/bottle, and $^{13}C_1$-propionate or $^{13}C_3$-propionate was less than 0.30 µmol/bottle.

Table 4.5 Fractional amounts of conversion of $^{13}C_3$-propionate and 2-$^{13}C_1$-propionate to acetate and butyrate after 8 h *in vitro* incubation in the absence of inhibitor and in the presence of BES or chloroform.

	$^{13}C_3$-propionate Mean fractional amounts (± SEM[a]; n = 2[b])		**2-$^{13}C_1$-propionate Mean fractional amounts (± SEM[a]; n = 2[b])**	
Inhibitors	f_{pa}	f_{pb}	f_{pa}	f_{pb}
Control	0.02629 (± 0.00036)	0.00037 (± 0.0000106)	0.02377 (± 0.00088)	0.00063 (± 0.00041)
BES	0.01334 (± 0.00024)	0.00030 (± 0.0000118)	0.01302 (± 0.00050)	0.00078 (± 0.00025)
$CHCl_3$	0.01243 (± 0.00047)	0.00029 (± 0.0000054)	0.01136 (± 0.00050)	0.00081 (± 0.00010)

[a]Standard error of the mean.

[b]No. of experimental replicates.

Table 4.6 Mean amounts of ^{13}C-labelled VFA (acetate, propionate and butyrate) formed due to VFA inter-conversion after 8 h *in vitro* incubation.

Mean (± SEM[a]) amounts of ^{13}C-labelled VFA formed (µmol/bottle) after 8 h of incubation (n = 2[b])							
$^{13}C_2$-acetate (30 mM added; 1800 µmol/bottle)							
Products	$^{13}C_1$-propionate (P^*)	$^{13}C_2$-propionate (P^{**})	$^{13}C_3$-propionate (P^{***})	$^{13}C_1$-butyrate (B^*)	$^{13}C_2$-butyrate (B^{**})	$^{13}C_3$-butyrate (B^{***})	$^{13}C_4$-butyrate (B^{****})
	2.13 (± 0.23)	37.80 (± 1.63)	0.72 (± 0.23)	2.16 (± 0.45)	81.35 (± 17.04)	2.80 (± 0.44)	37.67 (± 5.57)
$^{13}C_3$-propionate (15 mM added; 900 µmol/bottle)							
Products	$^{13}C_1$-acetate (A^*)	$^{13}C_2$-acetate (A^{**})	$^{13}C_1$-butyrate (B^*)	$^{13}C_2$-butyrate (B^{**})	$^{13}C_3$-butyrate (B^{***})	$^{13}C_4$-butyrate (B^{****})	
	1.59 (± 0.60)	17.25 (± 2.62)	0.66 (± 0.25)	1.40 (± 0.33)	0. 49 (± 0.06)	0.07 (± 0.02)	
$^{13}C_4$-butyrate (10 mM added; 600 µmol/bottle)							
Products	$^{13}C_1$-acetate (A^*)	$^{13}C_2$-acetate (A^{**})	$^{13}C_1$-propionate (P^*)	$^{13}C_2$-propionate (P^{**})	$^{13}C_3$-propionate (P^{***})		
	2.69 (± 0.41)	58.55 (± 19.35)	0. 25 (± 0.05)	1.00 (± 0.20)	–0.16 (± 0.23)		

[a]Standard error of the mean.

[b]No. of experiments.

The data summarised in Table 4.6 were then used to calculate the fractional amount of inter-conversions after 8 h *in vitro* incubation. The fractional amount of $^{13}C_2$-acetate converted to $^{13}C_2$-propionate (f_{ap}) and $^{13}C_2$- or $^{13}C_4$-butyrate (f_{ab}) was calculated according to equations (12) and (13) respectively. The amounts of $^{13}C_1$-propionate, $^{13}C_3$-propionate, $^{13}C_1$-butyrate and $^{13}C_3$-butyrate formed from $^{13}C_2$-acetate were small, as described above, so these were not used in calculations.

$$f_{ap} = P^{**}/A^{**} \quad (12)$$

$$f_{ab} = B^{**}/A^{**} \text{ or } 2 \times B^{****}/A^{**} \quad (13)$$

where P^{**} is the amount of $^{13}C_2$-propionate formed, B^{**} is the amount of $^{13}C_2$-butyrate formed, B^{****} is the amount of $^{13}C_4$-butyrate formed and A^{**} is the amount of $^{13}C_2$-acetate added (1800 μmol/bottle).

The fractional amount of $^{13}C_4$-butyrate converted to $^{13}C_2$-acetate (f_{ba}) and $^{13}C_2$-propionate (f_{bp}) was calculated according to equations (14) and (15) respectively. The amounts of $^{13}C_1$-acetate, $^{13}C_1$-propionate and $^{13}C_3$-propionate formed from $^{13}C_4$-butyrate were very small, as described above, so these were not used in calculations.

$$f_{ba} = A^{**}/(2 \times B^{****}) \quad (14)$$

$$f_{bp} = P^{**}/B^{****} \quad (15)$$

where A^{**} is the amount of $^{13}C_2$-acetate formed, P^{**} is the amount of $^{13}C_2$-propionate formed and B^{****} is the amount of $^{13}C_4$-butyrate added (600 μmol/bottle).

The fractional amounts of $^{13}C_3$-propionate converted to $^{13}C_2$-acetate (f_{pa}) and $^{13}C_3$-butyrate (f_{pb}) were calculated according to equations described earlier (8) and (9) respectively.

The fractional amount of $^{13}C_2$-acetate converted to $^{13}C_2$-propionate (f_{ap}) was 0.021 and to $^{13}C_2$- or $^{13}C_4$-butyrate (f_{ab}) was 0.045 (the value used for further calculations) and 0.042 respectively (Figure 4.14). This confirms that there was a small fractional amount of inter-conversion between acetate to propionate, whereas the conversion from acetate to butyrate was greater. The fractional amount of $^{13}C_4$-butyrate converted to $^{13}C_2$-acetate (f_{ba}) was 0.049, and the conversion of $^{13}C_4$-

butyrate to $^{13}C_3$-propionate was very small (f_{bp} = 0.0017). The fractional amount of $^{13}C_3$-propionate converted to $^{13}C_2$-acetate (f_{pa}) was 0.019, much greater than to $^{13}C_3$-butyrate (f_{pb} = 0.001). These data confirmed that the VFA inter-conversion between acetate and butyrate was greater (3 to 56-fold) than between acetate and propionate or between butyrate and propionate (or vice-versa). However, the VFA conversion between the three VFA was not numerically large in the present experiments. Even so, all these fractional amounts of VFA inter-conversions should be accounted for in the calculations of acetate formation from homoacetogenesis and butyrate formation from homobutyrogenesis.

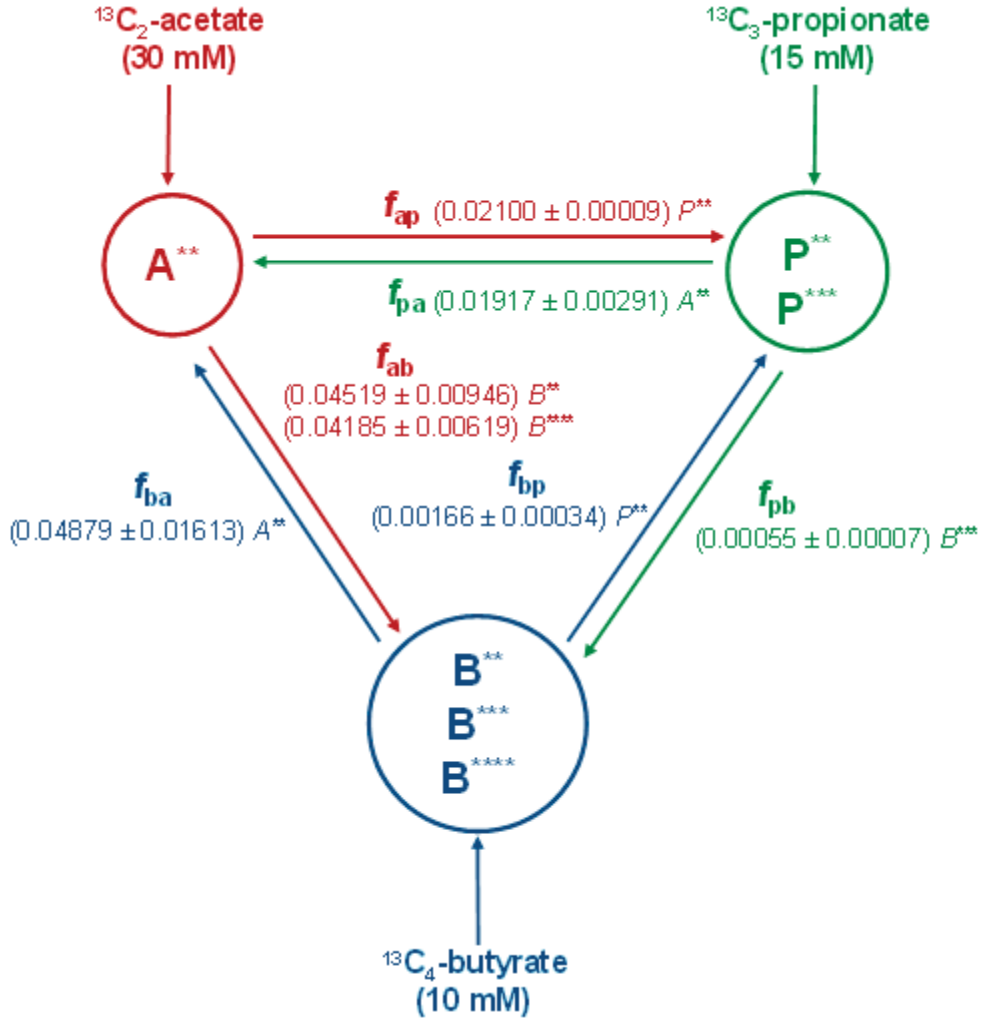

Figure 4.14 Three tracer substrate pathway model ($^{13}C_2$-acetate, $^{13}C_3$-propionate and $^{13}C_4$-butyrate) depicting label transfer during 8 h *in vitro* incubation of rumen fluid. The model describes the fractional amount of inter-conversion between acetate, propionate and butyrate. The fractional data are presented as means of two separate experiments ± standard error of the mean in bracket followed by the actual conversion product formed. A, acetate; P, propionate, B, butyrate; *, one carbon labelled; **, two carbons labelled; ***, three carbons labelled; ****, four carbons labelled.

4.4.4.1 Recalculating acetate formed from homoacetogenesis correcting VFA inter-conversions

The amount of acetate formed from homoacetogenesis estimated from label incorporation into acetate from $NaH^{13}CO_3$ in Experiment 1 was recalculated to account for VFA inter-conversion, using the fractional conversion factors calculated from the data derived from Experiment 4 (Figure 4.14). The model for the VFA inter-conversion is outlined in Figure 4.15.

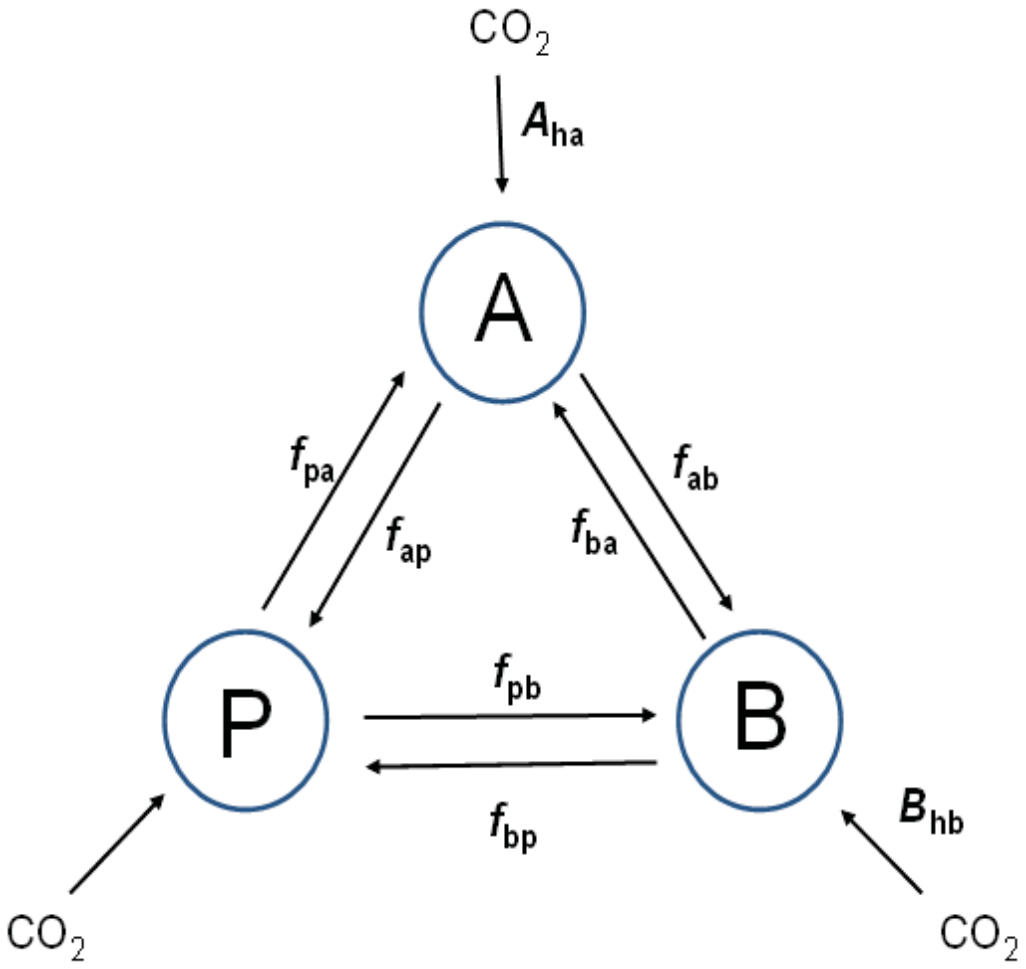

Figure 4.15 One tracer substrate pathway model ($NaH^{13}CO_3$) depicting label transfer during 8 h *in vitro* incubation of rumen fluid. The model describes the fractional amounts of inter-conversion between acetate, propionate and butyrate when $NaH^{13}CO_3$ is added and all the activities (VFA inter-conversion) are occurring simultaneously. A, acetate; P, propionate; B, butyrate; A_{ha}, acetate formation from homoacetogenesis; B_{hb}, butyrate formation from homobutyrogenesis.

The enrichment data from IRMS measurements combined with the VFA concentrations in the *in vitro* assays were used to calculate the amount of excess

labelled acetate (^{13}A), propionate (^{13}P) and butyrate (^{13}B) present, using equations described earlier (1), (2) and (3) respectively.

The quantity of ^{13}C-labelled acetate produced via homoacetogenesis ($^{13}A_{ha}$) was measured and corrected by subtracting labelled acetate that came from propionate ($f_{pa} \times {}^{13}P$) and butyrate ($f_{ba} \times {}^{13}B$) and adding labelled acetate that was converted (or lost) to propionate ($f_{ap} \times {}^{13}A$) and butyrate ($f_{ab} \times {}^{13}A$) to the amount of excess labelled acetate (^{13}A).

$$^{13}A_{ha} = {}^{13}A - f_{pa} \times {}^{13}P - f_{ba} \times {}^{13}B + f_{ap} \times {}^{13}A + f_{ab} \times {}^{13}A \quad (16)$$

where f_{pa} is the fractional amount of labelled propionate converted to labelled acetate (equation 8), f_{ba} is the fractional amount of labelled butyrate converted to labelled acetate (equation 14), and f_{ap} and f_{ab} are the fractional amounts of labelled acetate converted to labelled propionate and butyrate respectively (equation 12 and 13).

Finally, from the ratio excess of $^{13}CO_2$ measured via IRMS ($r^{13}CO_2$), the total quantity of acetate (unlabelled and labelled) produced via homoacetogenesis (A_{ha}) was calculated (equation 17).

$$A_{ha} = {}^{13}A_{ha}/r^{13}CO_2 \quad (17)$$

where $r^{13}CO_2$ is the ratio of excess of dissolved $^{13}CO_2$ relative to control without $NaH^{13}CO_3$ added (equation 18).

$$r^{13}CO_2 = (rCO_{2\ Lferm} - rCO_{2\ Uferm})/(rCO_{2\ Lferm} + 1) \quad (18)$$

where $rCO_{2\ Lferm}$ and $rCO_{2\ Uferm}$ represents the ratio of $^{13}C/^{12}C$ in CO_2 in the labelled versus unlabelled systems respectively after 8 h of incubation.

The amount of acetate formed from homoacetogenesis (uncorrected and corrected for VFA inter-conversion) was expressed as both the amount of acetate produced from homoacetogenesis in μmol/bottle (Figure 4.16a) and as percentages of total acetate produced ($A_{ha}/A \times 100$; Figure 4.16b). The amount of acetate from homoacetogenesis in the assays without inhibitor, after correction for VFA inter-conversion, now accounted for 15.1 μmol/bottle (Figure 4.16a) after 8 h of *in vitro* incubation. With addition of BES it increased significantly by 40.4% to 21.2

µmol/bottle ($p = 0.001$), whereas chloroform decreased it by 55.4% to 6.74 µmol/bottle ($p < 0.0001$), compared to assays without inhibitor. Therefore, after correcting the amount of acetate from homoacetogenesis for VFA inter-conversion with propionate and butyrate, the amount of homoacetogenesis was lower. VFA inter-conversion accounted for a decrease of only 2.03-3.40 µmol/bottle of in amount of acetate from homoacetogenesis in the assays without inhibitor and presence of BES or chloroform. At most, VFA inter-conversion accounted for 33.5% of the apparent measured $^{13}CO_2$ incorporation into acetate without correction, highlighting the need to take inter-conversion into account.

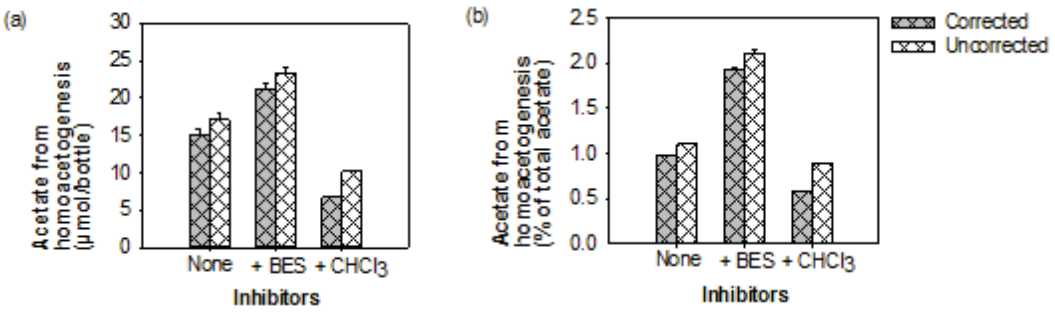

Figure 4.16 Amount of acetate (a) and percentage of acetate (b) produced from homoacetogenesis following incubation of sheep rumen contents *in vitro* after 8 h in the absence of inhibitor and presence of BES or chloroform, after correcting for VFA inter-conversion as well as uncorrected values. Data are presented as means ± standard errors of the mean ($n = 3$).

4.4.4.2 Recalculating butyrate from homobutyrogenesis using VFA inter-conversion corrections

The amount of butyrate from homobutyrogenesis in Experiment 1 was also corrected for VFA inter-conversion using the data in Figure 4.14. The quantity of ^{13}C-labelled butyrate produced via homobutyrogenesis ($^{13}B_{hb}$) was measured the same way as $^{13}A_{ha}$, i.e., it was measured and corrected by subtracting labelled butyrate that came from propionate ($f_{pb} \times {}^{13}P$) and acetate ($f_{ab} \times {}^{13}A$) and adding labelled butyrate that is converted (or lost) to propionate ($f_{bp} \times {}^{13}B$) and acetate ($f_{ba} \times {}^{13}B$) to the amount of excess labelled butyrate (^{13}B).

$$^{13}B_{hb} = {}^{13}B - f_{pb} \times {}^{13}P - f_{ab} \times {}^{13}A + f_{bp} \times {}^{13}B + f_{ba} \times {}^{13}B \quad (19)$$

where f_{pb} is the fractional amount of labelled propionate converted to labelled butyrate (equation 9) and f_{ab} is the fractional amount of labelled acetate converted

to labelled butyrate (equation 13). f_{ba} and f_{bp} are the fractional amounts of labelled butyrate converted to labelled propionate and acetate, respectively (equation 14 and 15).

Finally, from the ratio excess of $^{13}CO_2$ measured via IRMS ($r^{13}CO_2$; equation 18), the total quantity of butyrate (unlabelled and labelled) produced via homobutyrogenesis (B_{hb}) was calculated (equation 20).

$$B_{hb} = {}^{13}B_{hb}/r^{13}CO_2 \quad (20)$$

The butyrate formed from homobutyrogenesis (uncorrected and corrected for VFA inter-conversion) was expressed as both the amount of butyrate produced from homobutyrogenesis in µmol/bottle (Figure 4.17a) and as percentages of total butyrate produced ($B_{hb}/B \times 100$; Figure 4.17b). The amount of butyrate formed from homobutyrogenesis after correction was 1.85 µmol/bottle the assays without inhibitor after 8 h of *in vitro* incubation (Figure 4.17a). With the addition of inhibitor BES, it increased significantly by more than 100% to 3.78 µmol/bottle ($p = 0.002$) and with chloroform there was a trend towards an increase by 46.1% to approximately 2.71 µmol/bottle ($p = 0.086$) as compared to the assays without inhibitor. The VFA inter-conversion accounted for a decrease of only 0.33-0.81 µmol/bottle in the amount of butyrate from homobutyrogenesis in the assays without inhibitor and presence of BES or chloroform, and so was at most 27.0% of the activity measured without correction.

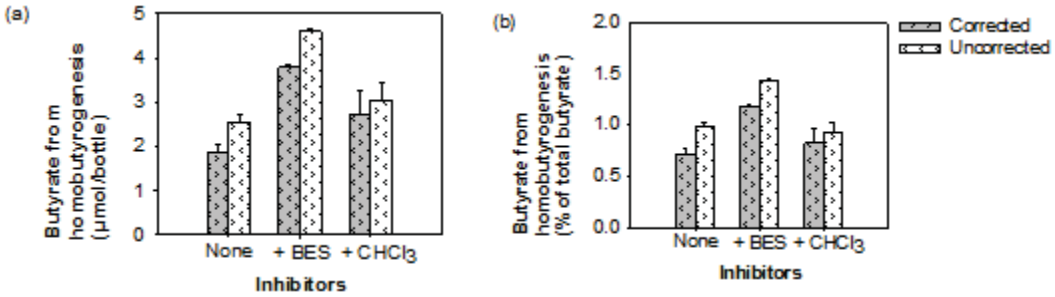

Figure 4.17 Amount of butyrate (a) and percentage of butyrate (b) produced from homobutyrogenesis following incubation of sheep rumen contents *in vitro* after 8 h in the absence of inhibitor and presence of BES or chloroform, after correcting for VFA inter-conversion as well as uncorrected values. Data are presented as means ± standard errors of the mean ($n = 3$).

4.4.4.3 Hydrogen concentration versus relative rate of hydrogen metabolism for homoacetogens

Theoretically, the relative rate of hydrogen metabolism for homoacetogens with a threshold concentration of hydrogen can be expressed as a function of the concentration of dissolved hydrogen (equation 21):

$$V = V_{max} \times (S - S_{min})/(K_s + (S - S_{min})) \quad (21)$$

where V is the relative rate of metabolism, V_{max} is the maximum rate, S is the hydrogen concentration, S_{min} is the minimum threshold hydrogen concentration and K_s is the half saturation constant (also referred to as Monod's constant; Caperon & Meyer 1972).

Assuming V_{max} to be 1, and using data from the literature where S_{min} for *Acetobacterium woodii* (a homoacetogen) is 383 nM dissolved hydrogen (Cord-Ruwisch *et al.* 1988) and the K_s value for *Acetobacterium woodii* is 710 μM dissolved hydrogen (Peters *et al.* 1998), the relative rates of metabolism of hydrogen by homoacetogens can be estimated relative to V_{max}. The headspace and dissolved hydrogen concentrations in *in vitro* may not be in equillibrium, and headspace hydrogen concentration may not always be a good way to calculate dissolved hydrogen (Wang *et al*. 2014). However, here it has been assumed that they are in equilibrium in order to allow an estimate of the rate of metabolism of homoacetogens to be made. Therefore, dissolved hydrogen concentrations were calculated from headspace hydrogen concentrations determined using GC data, based on a maximum possible 100% (v/v) hydrogen in the bottle headspace which equals 737 μM dissolved hydrogen at 39 °C (Janssen 2010). These values were then used in equation (21) to calculate relative rate of hydrogen metabolism for homoacetogens (V).

Figure 4.18a represents the calculated theoretical relative rates of hydrogen metabolism by homoacetogens as described above in equation (21). In the assays with no inhibitor, the hydrogen concentration was very low (< 1.2 μM), and the calculated relative rate of metabolism for homoacetogens was also very low (< 0.002; Figure 4.18b). This is consistent with the small amount of acetate formed from homoacetogenesis (Figure 4.16a). With addition of BES, the hydrogen concentration increased by 37.3-fold to approximately 46 μM at 8 h with inhibition

of methane production, and the estimated relative rate of metabolism for homoacetogens increased by 51.6-fold to 0.060 (Figure 4.18c). This may explain the increase in acetate formed from homoacetogens after 8 h inhibition with BES (Figure 4.16b). In the presence of chloroform, the hydrogen concentration increased by 39.0-fold to approximately 48 μM at 8 h, and the estimated relative rate of metabolism for homoacetogens also increased by 53.4-fold to 0.062 (Figure 4.18d). However, a decrease in acetate formed from homoacetogens was observed after 8 h in the presence of chloroform (Figure 4.16c). Therefore, although the hydrogen concentration was sufficient to allow an increase in homoacetogenesis, and was similar to that found when BES was added, the lack of response is consistent with the known inhibitory effects of chloroform on homoacetogens. This also adds evidence that the $^{13}CO_2$ incorporation into acetate was actually due to homoacetogenic activity.

The analyses presented here suggest that, with increases in hydrogen concentration during inhibition of methane production, the relative rate of metabolism of homoacetogens also increased and that there is potential for them to increase homoacetogenic activity over long-term inhibition of methane production. This is because the dissolved hydrogen concentrations after methanogen inhibition appear to be in a range that is sufficient to allow homoacetogens to grow with hydrogen. It is not known, of course, whether the observed homoacetogenesis is from growth with H_2 plus CO_2, or is due to acetate formation via the Wood-Ljungdahl pathway using electrons derived from fermentative metabolism.

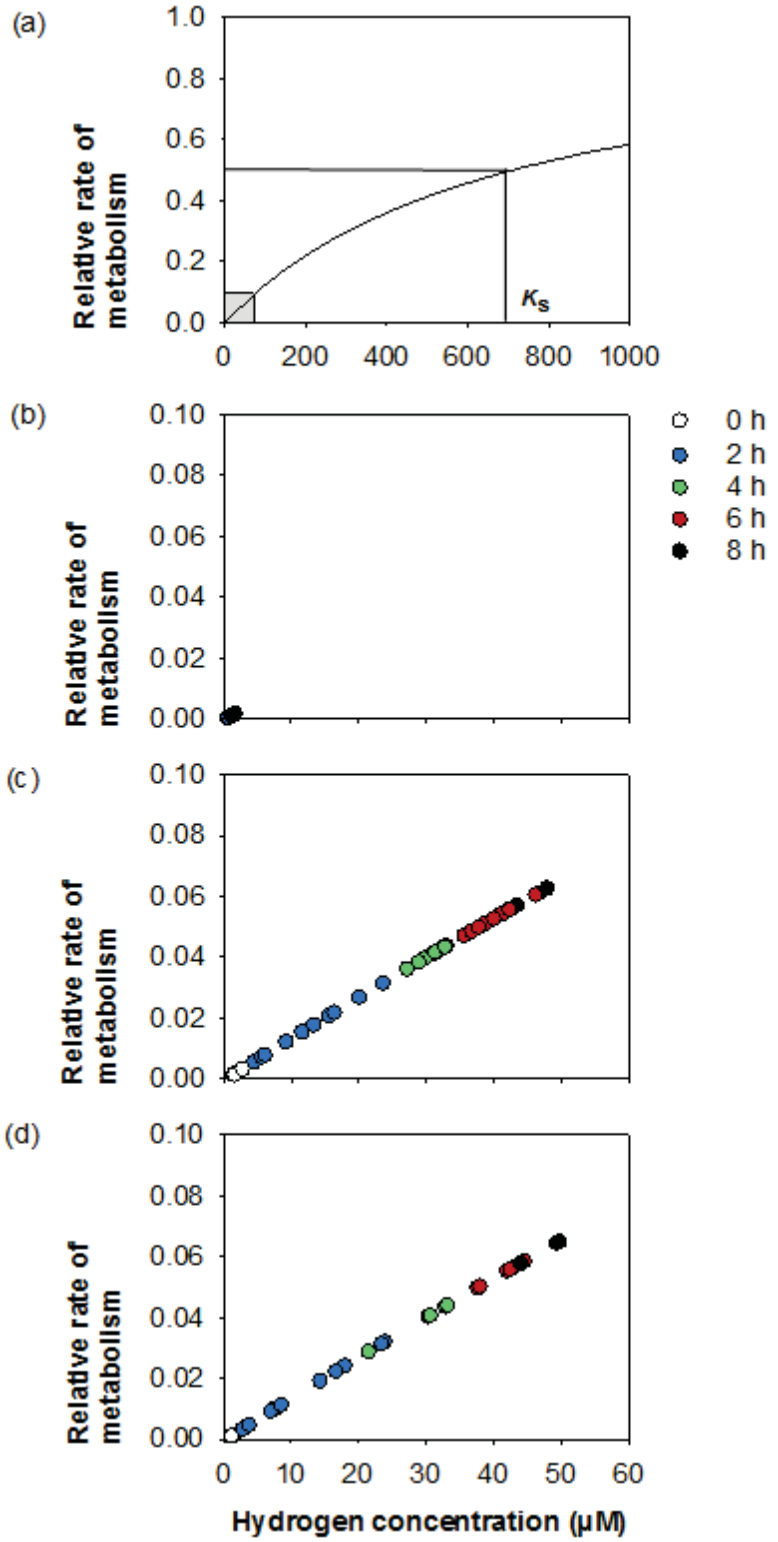

Figure 4.18 Theoretical relative rates of metabolism versus hydrogen concentration for homoacetogens (a) with K_s = 710 µM and S_{min} = 383 nM. The shaded area in (a) was then plotted using actual data points, i.e., dissolved hydrogen concentrations versus calculated relative rate of metabolism of homoacetogens in sheep rumen contents incubated *in vitro* in the absence of inhibitor (b) and in the presence of BES (c) or chloroform (d) over time, represented by symbols with different colours. Data points from all experimental replicates (n = 3) of each treatment are plotted.

4.4.5 Flow of electrons during short-term *in vitro* rumen fermentation (electron balance)

The majority of the hydrogen produced during rumen fermentation is utilised in the formation of methane (Czerkawski 1972) which maintains low concentrations of dissolved and gaseous hydrogen in the rumen. Once methanogenesis is inhibited, hydrogen that was utilised by methanogenesis might now be used by other processes occurring in the rumen such as homoacetogenesis. There will also be a shift in the rumen fermentation so that less hydrogen is formed, and electrons are used for the formation of reduced VFA like propionate, butyrate and valerate. Therefore, it is of interest to study the flow of hydrogen and electrons during rumen fermentation and to determine which alternative hydrogen- or electron-utilising processes become active in the absence of methanogenesis.

4.4.5.1 Calculations for electron balance

The electron balance, often called a (metabolic) hydrogen balance, was calculated from data produced after 8 h of *in vitro* rumen fluid incubation (Experiment 1) in the absence and presence of BES or chloroform. Note that a hydrogen balance might imply that hydrogen gas is used in the formation of some of the VFA formed. However, here it accounts for electrons used, in the form of NADH, reduced ferredoxin, hydrogen gas, or other electron carriers. These balances are theoretical, but help understand how fermentation changes under different conditions. The shift in the electron balance was calculated from the production and utilisation of electrons (written as reduced protons, or H) using following equations:

$$2H \text{ produced} = 2A_f + P_f + 4B_f + 3V_f \quad (22)$$

$$2H \text{ utilised} = 2P_f + 2B_f + 4V_f + 4A_{ha} + 10B_{hb} + 4M + H_2 \quad (23)$$

where *2H* (i.e., two reduced protons) produced is the amount of hydrogen that could be generated during the formation of the products accounted for equation 22 and *2H* utilised is the amount of hydrogen that could be used (or saved and so not produced) in the formation of the products (equation 23). A_f, P_f, B_f and V_f are acetate, propionate, butyrate and valerate formed from fermentation. M and H_2 are methane and hydrogen formed. A_{ha} and B_{hb} are the amount of acetate produced from homoacetogenesis and butyrate produced from homobutyrogenesis respectively and were calculated from equations (17) and (20) respectively after correction for

VFA inter-conversion. A_f and B_f were calculated by subtracting the amount of acetate formed from homoacetogenesis (A_{ha}) and butyrate formed from homobutyrogenesis (B_{hb}) from total amounts of acetate (A) and butyrate (B) produced, respectively (equation 24 and 25). All units are in moles.

$$A_f = A - A_{ha} \quad (24)$$

$$B_f = B - B_{hb} \quad (25)$$

The electron recovery was then calculated as the ratio between *2H* utilised and *2H* produced, and expressed as a percentage (equation 26):

$$2H\ \%\ \text{recovery} = 2H\ \text{utilised} \times 100/2H\ \text{produced} \quad (26)$$

These electron balance calculations are based on those of Demeyer and Van Nevel (1975) and Faichney *et al*. (1999), and have been further modified to account for hydrogen utilised in homoacetogenesis and homobutyrogenesis.

4.4.5.2 Proportion of electron utilisation accounted for by each metabolite

The proportion of electrons utilised was calculated for each metabolite formed as a proportion of total electrons utilised (*2H* utilised; Figure 4.19). In the the assays without inhibitor, 40.3% of the total *2H* was used in methane production, 0.33% in hydrogen production, 37.1% in propionate production, 14.1% in butyrate production, and 6.05% in valerate production. The amount of *2H* utilised in homoacetogenesis was around 1.67% and in homobutyrogenesis 0.51% (Figure 4.19a). Addition of BES *in vitro* resulted in a decrease in *2H* utilisation by methane formation to 2.14% of the total, and increases in *2H* being used in propionate production to 53.9%, butyrate to 22.0% and valerate to 9.50% (Figure 4.19b). Hydrogen also accumulated with inhibition of methane production and it accounted for 8.18% of *2H*. Electron utilisation by homoacetogenesis increased to 2.93% of *2H* with inhibition of methane production using BES and homobutyrogenesis accounted for 1.31%, both greater than in the assays without inhibitor (Figure 4.19a). Addition of chloroform *in vitro* had similar effects to BES, but *2H* utilisation by homoacetogenesis reduced to 0.94%, as chloroform inhibits homoacetogenesis, while homobutyrogenesis increased to 0.93% (Figure 4.19c) as compared to the assays without inhibitor. Therefore, inhibiting methane production using BES

appeared to increase the amount of hydrogen (or electrons) used by homoacetogenesis to 2.93% and chloroform reduced it to 0.94% over the short-term (8 h) incubation. Propionate seemed to be the most important electron sink, as *2H* utilisation by propionate increased from 37.1% in the assays without inhibitor to 53.9% or 56.4% in the presence of BES or chloroform, respectively.

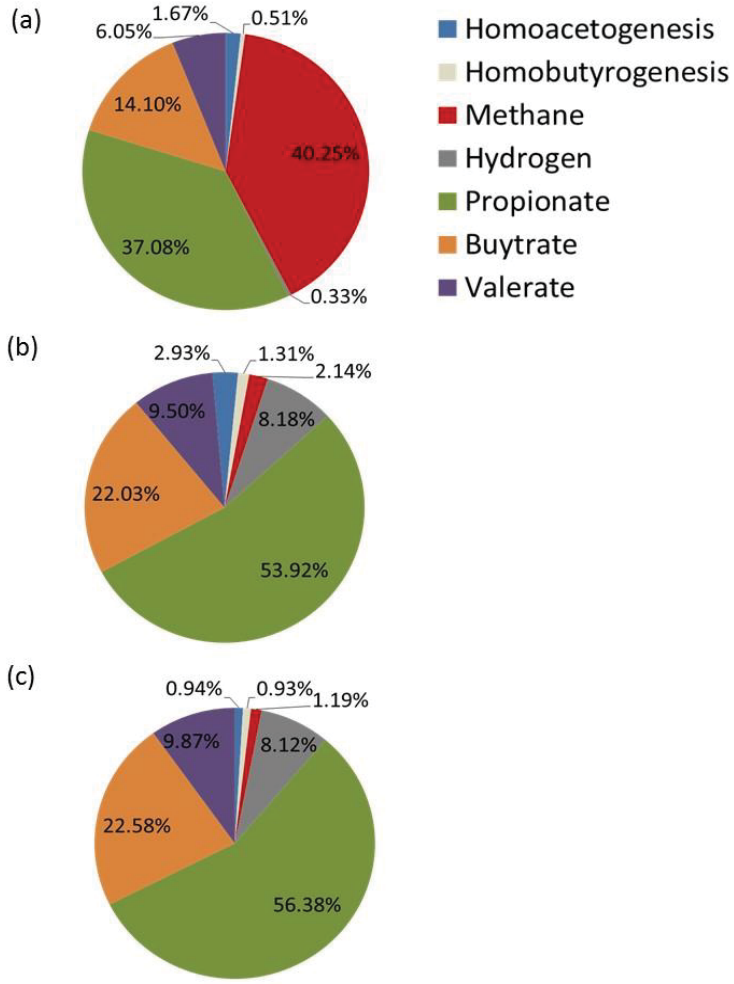

Figure 4.19 Electrons utilised (%) in production of various metabolites in sheep rumen contents incubated *in vitro* for 8 h in the absence of inhibitor (a) and in the presence of BES (b) or chloroform (c). Data are presented as means (n = 3).

4.4.5.3 Electron recovery

The electron recovery was calculated for the incubations in the absence of an inhibitor and in the presence of BES or chloroform according to equation 26. Approximately 74% electron recovery was found in control incubations (no inhibitor; Table 4.7). In the presence of BES, it decreased to 65% and with chloroform it decreased to 63%.

Table 4.7 Electron recovery in sheep rumen contents incubated *in vitro* after 8 h.

Inhibitors	**Mean electron recovery (%, ± SEM[a]; *n* = 3[b])**
None	73.6 ± 2.36
BES	65.4 ± 0.70
$CHCl_3$	62.6 ± 0.58

[a]Standard error of the mean.

[b]No. of experimental replicates.

4.4.6 Discussion

Based upon previous studies that investigated the effects of BES on rumen fluid *in vitro* (Martin & Macy 1985, Sauer & Teather 1987, Boccazzi & Patterson 1996, Choi *et al.* 2004, Lee *et al.* 2009) and chloroform on freshwater sediment samples (Scholten *et al.* 2000), anaerobic sludge digestion (Xu *et al.* 2010), anoxic rice field soil (Chidthaisong & Conrad 2000) and rumen fluid (Bauchop 1967, Lanigan 1972), methane formation from fresh rumen contents that were supplemented with fresh fermentable feed was expected to be inhibited (Table 4.1). Additionally, a change in fermentation processes and end products and increased homoacetogenesis was also anticipated. Addition of 10 mM BES decreased methane formation significantly by 95.8%, and hydrogen accumulated to 8.55% v/v of the total gas over 8 h. This is comparable to a study by Lee *et al.* (2009), where 5 mM BES resulted in 94.5-97.2% inhibition of methane production during 24 h rumen fluid incubation *in vitro*, and also resulted in accumulation of hydrogen to 3% of total gas compared to hydrogen in control incubations of less than 0.1% of total gas. Earlier studies also showed that 0.5 mM chloroform inhibited methane production by 100% during *in vitro* incubation of rumen fluid over 180 min (Bauchop 1967), and at concentrations as low as 0.005 mM complete but transient inhibition of methane production was observed over a 20 h incubation (Lanigan 1972). In the study reported in this chapter, addition of 2 mM chloroform resulted in 98.2% inhibition of methane formation after 8 h of incubation. Lee *et al.* (2009) also reported a decrease in total gas production with addition of BES, but the data presented in this chapter showed no decrease in total gas production with addition of either BES or chloroform, indicating that inhibition of methane had no severe effects on rumen fermentation and the extent of feed degradation *in vitro*. This

agreed with the results obtained with pure cultures, in which BES and chloroform did not affect growth of representative rumen bacteria (Chapter 3).

BES at 5 mM has been demonstrated to significantly decrease the molar proportion of acetate and increase the molar proportions of propionate and butyrate in the fermentation products after 48 h *in vitro* incubation of rumen fluid (Lee *et al.* 2009). During incubation of mesophilic sewage sludge with BES (50 mM) and chloroform (6.2 mM) over 48 days there was a decrease in acetate and associated increases in propionate and butyrate production (Xu *et al.* 2010). In the present study there was also a trend towards a decrease in acetate and a significant increase in propionate, as a proportion of the products, when methane formation was inhibited using BES or chloroform. The amount of valerate that accumulated increased and small amounts of isovalerate and isobutyrate were also increased significantly with inhibition of methane production. Lactate, formate and ethanol were also sometimes detected, but the amounts produced were very small (< 0.04 mmol/bottle). Overall, the total VFA production was not significantly affected over inhibition of methane production, but there were shifts in the mix of products.

Homoacetogenesis increased in rumen fluid when methane production was inhibited for 8 h using BES, which showed that there was potential to increase quickly in the absence of methanogenesis. This is consistent with a report that U-^{13}C-acetate formed during the incubation of bovine rumen contents *in vitro* with 100% (v/v) H_2 gas phase and $NaH^{13}CO_3$ (0.8 mM) in the presence of 5 mM BES increased from 0.02 mM at time zero to 0.24 mM after 45 h (Le Van *et al.* 1998), indicating a similar increase in homoacetogenesis. In the present study, the increase in homoacetogenesis can be attributed to an increase in dissolved hydrogen concentration due to inhibition of methane production with BES, rather than to the addition of 100% H_2 in the headspace, as in the study reported by Le Van *et al.* (1998). In the present study, hydrogen increased due to inhibition of methane production only, and was only 6.24% of the headspace gas, up from 0.15% in the assays without inhibitors. Using the gaseous hydrogen concentration, theoretical relative rates of metabolism of homoacetogens were calculated. These calculations indicated that at the hydrogen concentrations measured in the *in vitro* assays, homoacetogenic metabolism could have increased markedly. This suggests that the

increase in homoacetogenesis may have been in response to the increased hydrogen concentration. In the assays without inhibitors, the estimated rates of homoacetogenesis was very low, and the dissolved hydrogen concentrations were very close to the expected threshold for homoacetogenesis. Perhaps under these conditions homoacetogens are not using hydrogen, but growing with fermentative substrates and using the Wood-Ljungdahl pathway to dispose of electrons by additional acetate formation. That also results in the reduction of two CO_2 to acetate, which would lead in the formation of labelled acetate. It does seem that the activities measured were really homoacetogenesis, because the activities were greatly reduced by the addition of chloroform.

Chloroform is known to inhibit homoacetogenesis by inhibiting the carbon monoxide dehydrogenase/acetyl-CoA synthetase (CODH/ACS) complex of the homoacetogenic pathway (Ghambeer *et al.* 1971, Scholten *et al.* 2000), and resulted in inhibition of homoacetogenesis in freshwater sediments (Scholten *et al.* 2000), on rice roots (Conrad & Klose 2000) and during incubation of anaerobic sludge from a wastewater treatment plant (Xu *et al.* 2010). In the present experiment, small amounts of labelled acetate were still detected in the presence of chloroform, which might be due to resistance of some homoacetogen strains to chloroform as seen in pure culture studies against chloroform (Chapter 3). It has also been observed during incubation of bovine rumen fluid with $NaH^{14}CO_3$ in the presence of hydrogen and 50 μM chloroform, that there was a decrease in acetate synthesis from CO_2, but that it was not completely blocked (Prins & Lankhorst 1977).

Homoacetogens are also known to produce butyrate using CO_2 and H_2 (Zeikus *et al.* 1980, Kerby *et al.* 1983, Hensley *et al.* 2012, Schiel-Bengelsdorf & Dürre 2012), and this pathway of butyrate formation by homoacetogens can be referred to as homobutyrogenesis ($4CO_2 + 10H_2 \rightarrow CH_3CH_2CH_2COOH + 6H_2O$). This can be attributed to changes in the metabolism of homoacetogens depending upon environmental cues such as decreased pH, excess carbon monoxide (CO) or H_2, etc. (Rogers & Gottschalk 1993, Drake 1994, Drake *et al.* 2008). Members of the genera *Acetonema* and *Eubacterium* are known to be some of the homoacetogens producing butyrate along with acetate from CO_2 and H_2 (Schiel-Bengelsdorf & Dürre 2012). Apparent butyrate formation from homobutyrogenesis was also detected in the present study, and it seems to follow the same pattern as

acetate formation from homoacetogenesis, i.e., an increase in homobutyrogenesis occurs, when there is a decrease in methanogenesis caused by BES addition. This study appears to be to the first to detect and measure homobutyrogenesis in rumen contents. CO_2 incorporation into butyrate could be from the formation of two acetyl-CoA via homoacetogenesis followed by condensation and reduction to butyrate, or the condensation of one acetyl-CoA from homoacetogenesis and one from fermentation. This distinction was not investigated further. In both cases, CO_2 reduction to acetate has occurred.

During measurement of ^{13}C-label incorporation into acetate and butyrate from $^{13}CO_2$, a large amount of ^{13}C-label also appeared in propionate. The amount of ^{13}C-labelled propionate formed was approximately 11-fold greater than ^{13}C-labelled acetate in the assays with no inhibitor, and with addition of BES or chloroform it was approximately 9-fold or 21-fold that in acetate respectively. The amount of ^{13}C-labelled propionate formed was more than 45-fold greater than in labelled butyrate. Godwin *et al*. (2014) also reported appearance of ^{13}C-label in propionate during incubation of bovine rumen fluid and kangaroo fore-stomach contents with $NaH^{13}CO_3$ (Godwin *et al.* 2014). Therefore, it was important to determine how much of the ^{13}C-label that appeared in acetate or butyrate was due to VFA inter-conversion from propionate, as this might lead to an over-estimation of homoacetogenic and homobutyrogenic activity. It has been reported that label can also appear in other VFA when any labelled VFA is added to a system, due to VFA inter-conversion. During measurement of VFA production using a continuous infusion isotope dilution technique in the bovine rumen, it was found that propionate was converted to acetate and butyrate, although the percentage of inter-conversion is very low (less than 5% of the label; Esdale *et al.* 1968). Bergman *et al.* (1965) reported there was inter-conversion of VFA in the rumen of sheep fed dried grass cubes infused with 1-^{14}C-acetate, 1-^{14}C-propionate and 1-^{14}C-butyrate during a 5 h period. The inter-conversion between propionate and acetate or propionate and butyrate was considerably lower (Bergman *et al.* 1965). Leng and Brett (1966) also reported that the conversion of propionate to acetate or to butyrate or vice-versa was very small (Leng & Brett 1966). It has also been observed that infusion of 2-^{14}C-propionate into sheep rumen resulted in only 1.74% of total acetate and 8.1% of total butyrate produced in the VFA pool from propionate (Van Der Walt & Briel 1976). Similar results were observed in *in vitro* Experiment 3

reported here. A very small amount of VFA inter-conversion between propionate to acetate and butyrate was found in response to added $^{13}C_3$-propionate or 2-$^{13}C_1$-propionate. The amounts of inter-conversion were at most 0.96% of acetate and 0.21% of butyrate becoming labelled after 8 h of incubation in the absence of inhibitor. However, the amounts of homoacetogenesis and homobutyrogenesis were also small. The inter-conversion was accounted for and the apparent rates of homoacetogenesis and homobutyrogenesis corrected for this. When BES or chloroform were added along with $^{13}C_3$-propionate or 2-$^{13}C_1$-propionate, the amount of label that was transferred from propionate to acetate was decreased by approximately 48% and 56% respectively and not increased. The reason behind the decrease in the fractional amount of propionate converted to acetate with addition of inhibitors (BES or chloroform) is still unknown. However, this is in contrast with the increase in label found in acetate when the inhibitors were added in the presence of $^{13}CO_2$, and confirmed that most of the labelled acetate and butyrate originated from homoacetogenesis and homobutyrogenesis rather than VFA inter-conversion from labelled propionate.

It has been reported earlier that infusion of ^{14}C-acetate, ^{14}C-propionate and ^{3}H-butyrate into the rumen resulted in 40-50% of total butyrate produced being labelled from acetate and 6-13% of total acetate produced labelled from butyrate (Leng & Brett 1966). Van Der Walt and Briel (1976) reported 31.2% of total acetate coming from butyrate and 51.9% of total butyrate coming from acetate when the sheep rumen were infused with 1-^{14}C-acetate or 1-^{14}C-butyrate. Similar results were obtained during infusion of ^{14}C-labelled VFA into ruminants fed on different diets, with large amounts of inter-conversion between acetate and butyrate (Glinsky *et al.* 1976, Sharp *et al.* 1982, Bruce *et al.* 1987, Sutton *et al.* 2003). A three-compartment model, which represents VFA kinetics in the sheep rumen, has been developed based on studies of ^{14}C-acetate, ^{14}C-propionate and ^{14}C-butyrate infusion into sheep for 240 min (Nolan *et al.* 2014). That study reported VFA inter-conversion between the three major rumen VFA and showed that the major conversion was between acetate and butyrate (Nolan *et al.* 2014). In the study reported in this chapter (Experiment 4), the inter-conversion between all the three major VFA (acetate, propionate and butyrate) was determined during incubation of sheep rumen fluid *in vitro* and analysed using the three-tracer substrate model of Nolan *et al*. (2014). This allowed correction of acetate and butyrate inter-conversions to correct for

these in homoacetogenesis and homobutyrogenesis. The results were consistent with previous findings, that conversion between acetate to butyrate and vice-versa was greater (3 to 56-fold) than the conversion between propionate and acetate or propionate and butyrate. The fractional amount of VFA inter-conversion was very small in these 8 h *in vitro* incubations. Despite the fact that the fractional amounts of VFA inter-conversion were small, they were still accounted for in the calculations of the amount of acetate from homoacetogenesis and butyrate from homobutyrogenesis.

An increase in *2H* utilisation by homoacetogenesis was observed during inhibition of methane production with BES to 2.93% as compared to 1.67% in assays with no inhibitor. The increase in homoacetogenesis was, however, much lower than could have been expected from 41.2-fold increase in hydrogen concentration. Addition of chloroform decreased it to 0.94%, as chloroform is known to partially inhibit homoacetogenesis. A decrease in the percentage of electron recovery was observed with addition of BES or chloroform. Such a decrease in electron (hydrogen) recovery has also been reported during *in vitro* incubation of rumen fluid in the presence of additives (Ungerfeld *et al.* 2003) and the methanogen inhibitor bromochloromethane (Goel *et al.* 2009). Electrons can also be utilised in the production of various other fermentation end products such as ethanol, lactate and formate, etc. (Chalupa 1977, Ungerfeld *et al.* 2003), but these were very small and undetectable in the present study. It is not clear where the electrons are used, and why the recoveries are incomplete. However, such incomplete electron recoveries are widely reported.

4.5 Summary and perspectives

The short-term *in vitro* assay (Experiment 1) helped to validate and test the impact of BES and chloroform on *in vitro* rumen fermentation. Methane production was inhibited in the presence of BES or chloroform, but the extent of fermentation, based on total gas production, appeared to be unaffected by BES or chloroform addition. This experiment was also a useful development of methods to determine formation of ^{13}C-acetate and ^{13}C-butyrate from $^{13}CO_2$ during *in vitro* rumen fermentation. Incorporation of $^{13}CO_2$ into ^{13}C-acetate and ^{13}C-butyrate confirmed the occurrence of rumen homoacetogenesis and homobutyrogenesis in fresh sheep rumen fluid incubated *in vitro*. Homoacetogenesis and homobutyrogenesis

increased slightly when methane formation was inhibited using BES for 8 h, which shows that there is potential for homoacetogenesis and homobutyrogenesis to increase. Homoacetogenesis and homobutyrogenesis decreased with the addition of chloroform, which inhibits methanogens and partially homoacetogens (Conrad & Klose 2000, Scholten *et al.* 2000, Xu *et al.* 2010; see also Chapter 3). Using chloroform as an additional control confirmed that the label incorporation into acetate or butyrate was due largely to homoacetogenesis and homobutyrogenesis. Formation of butyrate by homobutyrogenesis via the activity of ruminal homoacetogens was also detected for the first time and it followed the same pattern as acetate formation from homoacetogenesis. During *in vitro* inhibition of methane with BES, the amount of *2H* utilised in homoacetogenesis increased to 2.93%, from 1.67% in assays with no inhibitor, which implies that there is potential for homoacetogenesis to increase. However, the increase in homoacetogenesis was much lower than could have been expected from the 41.2-fold increase in hydrogen concentration. It will be interesting to see what the impact of inhibition of methane production is on homoacetogenesis over a longer period, and whether its contribution increases further, or if this is a transient immediate response to a hydrogen concentration increase.

Experiment 2 showed that there were no major effects on gas production caused by adding increased concentrations of VFA (30 mM acetate, 15 mM propionate and 10 mM butyrate) to the *in vitro* rumen fermentation, although higher concentrations did have some inhibitory effects. Therefore, concentrations of acetate (30 mM), propionate (15 mM) and butyrate (10 mM) were selected to study VFA inter-conversion in Experiments 3 or 4. Experiments 3 and 4 measured the fractional amounts of VFA inter-conversion between acetate, propionate and butyrate. Although the fraction of inter-conversion between the three VFA was not large, the data were still used to correct calculations of the amount of acetate and butyrate formed from homoacetogenesis and homobutyrogenesis. BES (and chloroform) decreased the fraction of propionate converted to acetate and butyrate, in contrast to the increase in $^{13}CO_2$ incorporation into acetate and butyrate caused by BES, confirming that most of the labelled acetate and butyrate originated from homoacetogenesis and homobutyrogenesis rather than VFA inter-conversion. The contribution to total electron utilisation by homoacetogenesis and

homobutyrogenesis increased over short-term inhibition of methane production using BES, from 1.67% to 2.93% and 0.51% to 1.31% respectively.

The short-term *in vitro* assay developed here (Experiment 1) to investigate the effects of inhibiting methanogenesis will be used in an *in vitro* semi-continuous rumen fermentation experiment (Chapter 5) and in an *in vivo* trial (Chapter 6). Rumen contents can be taken at different times after inhibition, and the amount of homoacetogenesis and homobutyrogenesis measured via incorporation of $^{13}CO_2$ into ^{13}C-acetate and ^{13}C-butyrate, employing BES and chloroform as additional controls in the assay. The amount of acetate from homoacetogenesis and butyrate from homobutyrogenesis will be corrected for VFA inter-conversion using the equations described in Experiment 4, and the data summarized in Figure 4.14. The fractional conversion factors can be applied to the amounts of labelled product formed in incubations to correct for inter-conversion.

Chapter 5

Effect of inhibition of methanogenesis on fermentation and homoacetogenesis in sheep rumen fluid incubated in an *in vitro* serial batch fermentation experiment

5.1 Introduction

Methanogens are a characteristic part of the normal rumen ecosystem, and use H_2 and CO_2 to form methane. In various other gut microbial ecosystems, such as termites (Breznak 1994), pigs (De Graeve *et al.* 1994), the human colon (Wolin & Miller 1994), rabbits (Piattoni *et al.* 1996) and newly born lambs (Morvan *et al.* 1994), homoacetogens, organisms that convert H_2 and CO_2 to acetate, pre-dominate over methanogens *in vivo*. *In vitro* studies have been conducted to study the activity of homoacetogens in ruminal systems, to understand their role and their potential to displace or take over from methanogens. There have been a number of studies investigating the role of homoacetogens during *in vitro* incubation of rumen fluid, by addition of homoacetogens in the absence of methanogens or in the presence of feed additives.

Addition of the ruminant feed additive *Saccharomyces cerevisiae* to a co-culture experiment with the homoacetogen *Blautia* sp. Ser8 and the methanogen *Methanobrevibacter* sp. MF_2 stimulated hydrogen utilisation and acetate production by the homoacetogen under an artificially high partial pressure of hydrogen (Chaucheyras *et al.* 1995). In contrast, incubation of ruminal contents *in vitro* over 24 h with hay in the presence of casein hydrolysate and mucin, with and without added hydrogen, did not result in increased acetate formation from fermentation or enhanced reductive acetogenesis (Demeyer *et al.* 1996). *Blautia producta* U1, a homoacetogen, had no significant effect on methane or volatile fatty acid (VFA) production when added to an *in vitro* system containing rumen fluid, but when added together with the methanogen inhibitor BES, there was a decrease in methane production and an increase in acetate production from fermentation and an increase in hydrogen consumption (Nollet *et al.* 1997). Acetate formation from CO_2 was not measured. Incubation of rumen contents with cell-free supernatant of *Lactobacillus plantarum* 80 alone, or along with *Blautia producta*, reduced methane production with no hydrogen being accumulated and increased acetic acid

production from fermentation in VFA pool, but these effects reduced over long-term *in vitro* incubation and disappeared *in vivo* (Nollet *et al.* 1998). There was no evidence of reductive acetogenesis occurring. In another *in vitro* study, six homoacetogenic bacteria were incubated with rumen fluid in the presence or absence of the methanogen inhibitor, BES. In the absence of BES, only two acetogens (*Eubacterium limosum* strain Eggerth and *Blautia* sp. Ser5) decreased methane production by 5% after 24 h of incubation. When BES was added along with these two homoacetogens, methane formation was inhibited further and acetate formation increased. It is unknown if acetate came from fermentation or from homoacetogenesis. However, this led to the conclusion that adding homoacetogens alone under normal conditions did not decrease methane production substantially or increase acetate production, unless the methanogens were inhibited (Lopez *et al.* 1999). In another *in vitro* rumen fermentation experiment, the homoacetogen strain TWA4 added along with a *Saccharomyces cerevisiae* fermentation product (XP) enhanced total acetate formation, but methanogenesis was also increased (Yang *et al.* 2015). However, these studies did not differentiate the acetate derived from homoacetogenesis from acetate derived from carbohydrate fermentation. So, the contribution of acetate formation from homoacetogenesis was not very clear. A study by Le Van *et al*. (1998) did confirm the enhancement of acetate formation from H_2 and CO_2, in incubations when methane production was inhibited by BES and with addition of the rumen homoacetogen *Acetitomaculum ruminis* 190A4. These were added to rumen contents in the presence of $NaH^{13}CO_3$, and this resulted in increased formation of ^{13}C-labelled acetate, attributed to H_2/CO_2-utilising homoacetogenic activity (Le Van *et al.* 1998).

It seems that it is necessary to inhibit methanogenesis in order to direct more hydrogen towards homoacetogenesis. In the studies described above, homoacetogens were added to *in vitro* systems along with the methanogen inhibitor BES or a feed additive over short incubation periods, to study if the added homoacetogens or feed additives could enhance acetate production. In this chapter, experiments are reported in which methane formation was inhibited during serial transfer of *in vitro* incubations. These experiments explored if hydrogen accumulation caused by inhibition of methanogenesis could stimulate the resident homoacetogens to increase acetate production. This is in contrast to other studies in which the H_2 concentration was artificially elevated (Chaucheyras *et al*. 1995, Nollet

et al. 1997, Le Van *et al*. 1998). It was shown in Chapter 4 that the hydrogen concentrations in *in vitro* systems, in which methanogens were inhibited, were high enough to allow homoacetogens to be active. In this study, the activity of resident homoacetogens was assessed in the absence of methanogenesis, using the methanogen-specific inhibitor acetylene (see Chapter 3 for details on the selection of inhibitors). Although BES has been used as a methanogen inhibitor in a number of *in vitro* rumen fluid incubation studies (Martin & Macy 1985, Sauer & Teather 1987, Choi *et al.* 2004, Lee *et al.* 2009), its effects have been reported to wear off with time, due to adaptation of methanogens to BES in the sheep rumen (Immig *et al.* 1996) and in pure culture studies (Ungerfeld *et al.* 2004). Acetylene has been shown to inhibit methanogenesis during *in vitro* incubation of anaerobic paddy soils (Raimbault 1975), lake sediments (Macgregor & Keeney 1973) and sheep rumen fluid (Elleway *et al.* 1971). Acetylene completely inhibited methane production during a batch culture experiment using cow rumen fluid over five days (M. Tavendale, personal communication). Therefore, this inhibitor was used as the primary inhibitor of methanogenesis.

5.2 Objectives

The overall aim of the experiment was to determine if homoacetogenic activity increased during inhibition of methanogenesis in an *in vitro* serial batch fermentation experiment. To achieve this goal, sheep rumen fluid was incubated in the presence and absence of acetylene using an *in vitro* serial batch fermentation system. Serial transfer was carried out to provide fresh substrate and buffer every 12 h, as substrate and buffer nutrients are used by microbes. This also resulted in a partial removal of fermentation end-products to allow the fermentation and microbial growth to perpetuate efficiently. Acetylene was added every 12 h to inhibit methane production completely. Hence, the *in vitro* serial batch fermentation system allowed fermentation to be followed over time, while measuring the formation of various fermentation end products and tracking the changes in the microbial community composition. Homoacetogenesis was assessed using the short-term *in vitro* homoacetogenesis assay developed in Chapter 4, both prior to and at the end of the incubation of rumen contents with acetylene and no inhibitor over 108 h (nine serial transfers). In that assay, $^{13}CO_2$ incorporation into acetate was measured to estimate homoacetogenesis. Parallel short-term assays were also

conducted, adding BES to determine if there was additional potential for homoacetogenesis to increase, and chloroform, which should reduce rather than increase homoacetogenesis.

The objectives of the work reported in the present chapter were:

- To inhibit methane production using acetylene in sheep rumen fluid incubated *in vitro* in a serial batch fermentation system transferred nine times over 108 h
- To measure changes in the VFA profile during the *in vitro* serial batch fermentation in the presence and absence of methanogenesis, and measure other fermentation variables such as total gas, methane and hydrogen production
- To determine the changes in archaeal and bacterial community composition during *in vitro* serial batch fermentation
- To measure homoacetogenesis employing the short-term *in vitro* homoacetogenesis assay prior to (on fresh rumen fluid) and after 108 h inhibition of methanogenesis
- To study the contribution of homoacetogenesis and other alternative hydrogen or electron sinks to the flow of metabolic hydrogen or electrons before (on fresh rumen fluid) and after 108 h inhibition of methanogenesis with acetylene

5.3 Summary of materials and methods

An outline of the experiment along with variables measured is illustrated in Figure 5.1.

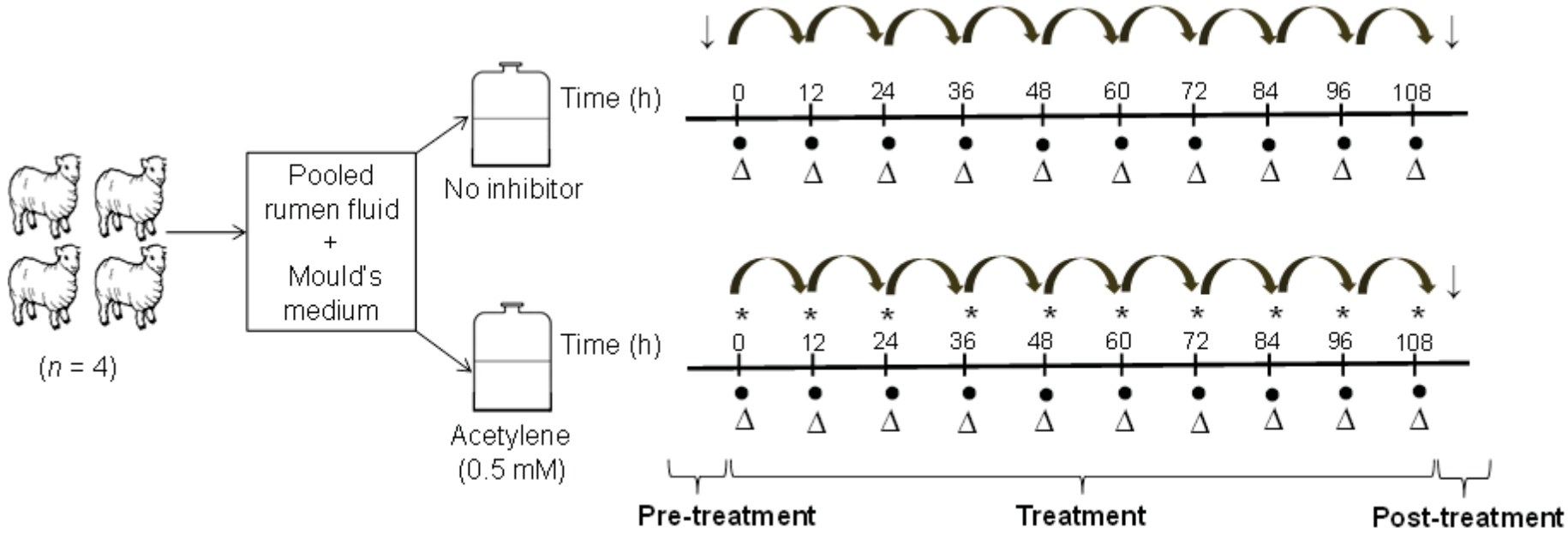

Figure 5.1 Overview of the *in vitro* serial batch fermentation experiment using sheep rumen fluid.

* Bottles received acetylene.

● Methane, hydrogen and total gas measured by gas chromatography (GC).

Δ Liquid fermentation samples were collected to determine VFA concentrations and microbial community structure (archaeal and bacterial 16S rRNA gene numbers, and relative abundance of different taxa).

↓ Short-term *in vitro* assay to measure homoacetogenesis as described in Chapter 4.

↷ Transfer of inoculum (20 ml; 33%) from previous bottle every 12 h into new bottle containing 0.6 g of dried ryegrass and 40 ml of fresh Mould's medium.

5.3.1 Rumen fluid sampling and *in vitro* incubation

Fistulated sheep (n = 4) fed on pasture diet were housed in pens in the morning (9 a.m.) and rumen fluid (200 ml) was collected from each sheep. The rumen fluid was then filtered through cheese cloth of approximately 1-mm mesh size (Stockinette; Cirtex Industries, Thames, New Zealand). The filtrate obtained was pooled together and diluted five-fold (v/v) in Mould's medium at pH 6.5, and maintained at 39 °C under a CO_2 atmosphere (Mould *et al.* 2005). Sixty millilitres of this was added to 120 ml volume serum bottles that each contained 0.6 g of dried ryegrass and a magnetic stirring bar, and had been held at 39 °C. Acetylene (0.5 mM) was added to some bottles (n = 8), whereas others received no inhibitor (n = 8). The bottles were sealed with rubber stoppers and incubated at 39 °C in a shaking incubator coupled to a GC system that continuously measured methane, hydrogen and total gas production (Muetzel *et al.* 2014). After every 12 h, 20 ml of the contents (33%) were taken from each bottle while keeping the bottles over a magnetic stirrer, and added to a new bottle containing 0.6 g of dried ryegrass, a magnetic stirring bar, and 40 ml of fresh Mould's medium maintained at 39 °C under a CO_2 atmosphere. The bottles receiving an acetylene-treated inoculum received acetylene (0.5 mM) again and bottles receiving no inhibitor inoculum received no inhibitor. Serial transfers were repeated every 12 h for 108 h, for a total of 9 transfers. Detailed descriptions of Mould's medium, acetylene preparation, and other methods can be found in Chapter 2.

5.3.2 Sampling from *in vitro* bottles and homoacetogenesis assay

Liquid samples (2 ml) were collected from each bottle every 12 h for VFA and microbial community analysis just prior to serial transfer (Figure 5.1, marked Δ). To measure homoacetogenesis at the beginning of the experiment (pre-treatment), fresh rumen fluid from the four sheep was diluted with Mould's medium, amended with 0.6 g ryegrass, and incubated for 8 h to measure homoacetogenesis using the assay developed in Chapter 4, measuring incorporation of $^{13}CO_2$ into ^{13}C-acetate, and adding BES or chloroform as additional controls. These inhibitors were used as additional checks to confirm that homoacetogenesis is due to incorporation of $^{13}CO_2$ into ^{13}C-acetate, which is not expected to be inhibited by BES, but should be at least partially inhibited by the addition of chloroform which is a known inhibitor of both methanogenesis and homoacetogenesis. BES was added at 3 mM and

chloroform at 100 μM concentrations. After 108 h of serial batch transfer, 20 ml of acetylene-treated inoculum as well as non-inhibited inoculum was taken and incubated in the same short-term assay to measure homoacetogenesis again. Other fermentation variables such as VFA, methane, hydrogen and total gas were also measured during this short-term *in vitro* homoacetogenesis assay at the pre- and post-treatment time points (Figure 5.1 marked ↓).

5.3.3 Analysis of samples

The liquid fermentation samples were analysed for the following:

- VFA by gas chromatography with a flame ionisation detector (GC-FID) and high-performance liquid chromatography (HPLC)
- microbial community structure was analysed for archaeal and bacterial 16S rRNA gene numbers by quantitative real-time PCR (qPCR), and the relative abundance of taxa assessed by 454 pyrosequencing of 16S rRNA genes
- homoacetogenesis pre-treatment (0 h) and after 108 h was measured employing the short-term *in vitro* homoacetogenesis assay via incorporation of $^{13}CO_2$ into ^{13}C-acetate (Chapter 4), and samples from that short-term assay were analysed using gas chromatography coupled with isotope ratio mass spectrometry (GC-IRMS) and solid-phase microextraction (SPME). Additional fermentation variables such as methane, hydrogen, total gas (via GC) and VFA (via GC-FID) were also measured

5.3.4 Statistical analysis of data from *in vitro* serial batch fermentation experiment

For the *in vitro* serial batch fermentation experiment, two treatments (no inhibitor and acetylene) with 8 experimental units (bottles) each were repeatedly observed and measured over 9 time points (12 to 108 h). There were several responses and each was analysed separately.

5.3.4.1 Repeated measures ANOVA

A *repeated measures ANOVA* with one experimental factor (2 treatments; no inhibitor and acetylene) and one repeated factor (9 time points, hours) was considered. Bottles were treated as genuine replications. Linear mixed effects models with Treatment, Time (h) and Treatment:Time (h) (interaction) as fixed effects and 'Bottle' as random effect were utilized with a covariance structure for

the random effect that accounts for the repeated nature of measurements made on each bottle. AIC (Akaike information criterion) was utilised to choose the optimal covariance structure. All analyses used 'autoregressive process of order 1' as a suitable choice with respect to the AIC criterion. P-values for paired comparisons were adjusted by the Bonferroni method. The significance of all effects were set at 5% ($p < 0.05$), and the effects with p-values more than 5% and less than 10% were regarded as a trend towards an increase or decrease ($p = 0.05$ to 0.10).

5.3.4.2 Transformations

When 'ANOVA assumptions' are not satisfactory when analysing the raw data (usually via examination of model residuals), the alternative is to base the inference on the transformed data (and/or use resampling methods such as the "permutation tests", also sometimes known as randomization tests). In this study, simple transformations such as SQRT (square root) and LOG (logarithm) were attempted. For those variables meeting the ANOVA assumptions only marginally, permutation tests (with 5000 randomizations) were evaluated and if the resulting significance of the various effects were very similar to those of the usual ANOVA, the results of the ANOVA were retained; otherwise, the permutation test results were retained.

5.3.4.3 Software

All analyses were carried out using the R software version 3.3.0 and packages such as *nlme* (and its *lme* function) and *predictmeans* (Pinheiro & Bates 2000, R Core Team 2016).

5.3.4.4 Principal component analysis

Principal component analysis (PCA) was based on Eigen-analysis of a correlation matrix using Minitab software (version 17; Minitab Inc., Pennsylvania, USA). The inputs were the relative abundance data for each genus. Where taxa were not be defined below the family or order level, those groups were used in the analysis as if they were genera.

5.3.5 Statistical analysis of data from short-term *in vitro* homoacetogenesis assay

Data were compared using one way ANOVA to compare treatment followed by Dunnett's multiple comparison with control using the Minitab software analysis tool (version 17; Minitab Inc., Pennsylvania, USA).

5.4 Results and discussion

Prior studies with pure cultures of methanogens and *in vitro* rumen fluid incubation indicated that acetylene inhibits methane production. Therefore, in the present study, it was expected that methane formation would be inhibited during the *in vitro* serial batch fermentation in the presence of acetylene, and that there would be changes in fermentation products and microbial community structure. It was also hypothesised that there would be an increase in homoacetogenesis due to inhibition of methanogens and the associated increase in the concentration of hydrogen available to homoacetogens (Table 5.1). This is based on the observation that there was an increase in homoacetogenesis after a short-term inhibition of methanogenesis with BES, and that this could have been explained by a potential increase in activity of homoacetogens due to increased hydrogen concentrations (Chapter 4).

Table 5.1 Expected effects in the serial batch *in vitro* fermentation over 108 h.

Inhibitor	Effects
No inhibitor	Normal methanogenesis and homoacetogenesis
Acetylene (0.5 mM)	Inhibit methanogenesis and increase homoacetogenesis

5.4.1 Acetylene inhibited methane production during *in vitro* serial batch fermentation

In the non-inhibited incubations, the amount of methane produced was initially 0.26 mmol/bottle (Figure 5.2a). Over time this decreased significantly ($p < 0.0001$) by 52.5% until, after 60 h, 0.12 mmol/bottle was being produced, and this remained more-or less constant thereafter. This may be because the microbial community changed in response to the new *in vitro* environment imposed by serial transfer every 12 h. The amount of hydrogen produced was less than 0.01 mmol/bottle (0.21% v/v of total gas) at 12 h, and increased significantly ($p < 0.0001$) by 3.23-fold to 0.024 mmol/bottle (1.04% v/v of total gas) over time in the serial transfers with no inhibitor. The total gas produced also decreased significantly ($p < 0.0001$) by 14.2% from 2.66 mmol/bottle at 12 h to 2.28 mmol/bottle at 108 h. In the presence of acetylene, the amount of methane produced decreased significantly ($p = 0.0001$) by 99.4% over 108 h (Figure 5.2b) of serial transfer as compared to the

series with no inhibitor (Figure 5.2a). The amount of hydrogen produced in the presence of acetylene was high at the beginning (12 h), at 0.13 mmol/bottle (5.42% v/v of total gas), but started decreasing significantly ($p < 0.0001$) over time by 54.0% to 0.058 mmol/bottle at 108 h (2.76% v/v of total gas). The amount of total gas produced in the presence of acetylene decreased significantly ($p < 0.0001$) by 7.59%, as compared to the series with no inhibitor, after 108 h.

The mean amounts of methane, hydrogen and total gas in the absence of inhibitor, and presence of acetylene during *in vitro* serial transfer over time are provided in Appendix 2 (Table A.2.1).

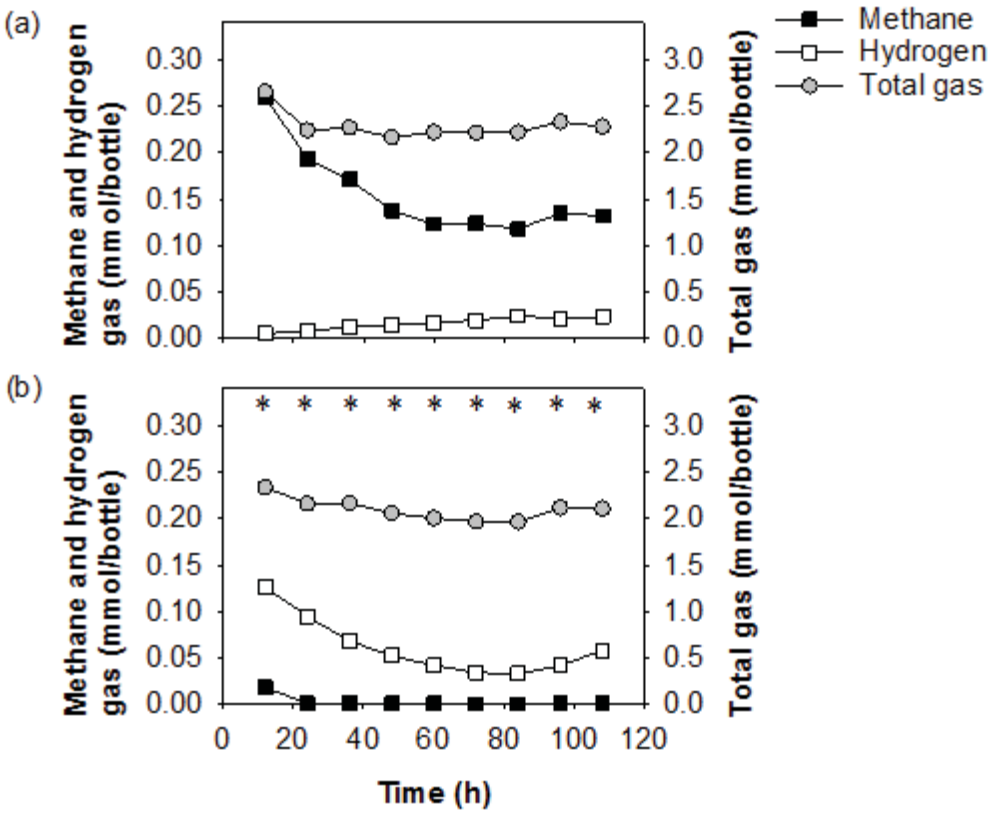

Figure 5.2 Methane, hydrogen and total gas produced by sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor (a) and in the presence of acetylene (b) over time. The * marks the times the serial transfers occurred and the bottles received acetylene. Data are presented as means ± standard errors of the mean, which are smaller than the symbols ($n = 8$).

5.4.2 Changes in volatile fatty acid profile of rumen fluid in the presence of acetylene

There were changes in the VFA produced following 12 h of incubation. In the incubations without an inhibitor, acetate, which was high at 12 h (1.77 mmol/bottle), decreased significantly ($p < 0.0001$) by 37.4% during serial transfer to 1.11 mmol/bottle at 108 h (Figure 5.3a). Propionate increased significantly ($p < 0.0001$) by 25.8% from 0.75 mmol/bottle at 12 h to 0.94 mmol/bottle at 108 h, and butyrate decreased significantly by 54.4% ($p < 0.0001$) from 0.34 mmol/bottle (12 h) to 0.15 mmol/bottle (108 h). In the presence of acetylene (Figure 5.3b), the amounts of acetate ($p = 0.0001$) and butyrate ($p = 0.0001$) produced decreased by 21.3% and 68.1% respectively, whereas propionate increased significantly ($p = 0.0001$) by 37.7% after 108 h as compared to the incubations with no inhibitor (Figure 5.3a). The amount of acetate produced was 0.87 mmol/bottle, propionate 1.29 mmol/bottle and butyrate 0.049 mmol/bottle at 108 h.

In the incubations without an inhibitor, acetate ($p < 0.0001$) and butyrate decreased significantly ($p < 0.0001$) by 20.6% and 42.3% respectively, whereas propionate increased significantly ($p < 0.0001$) by 60.0% as a proportion of total VFA over time during the serial transfers at 108 h as compared to 12 h (Figure 5.3c). In the presence of acetylene at 108 h, acetate ($p = 0.0001$) and butyrate ($p = 0.0001$) decreased by 18.9% and 67.9 % respectively, and propionate ($p = 0.0001$) became proportionally even more significant (increased by 43.2%) among the total VFA formed over time as compared to the incubations with no inhibitor at 108 h. Therefore, while there were changes in the VFA produced without acetylene, there was a marked change in the VFA profile towards greater propionate formation with inhibition of methane production.

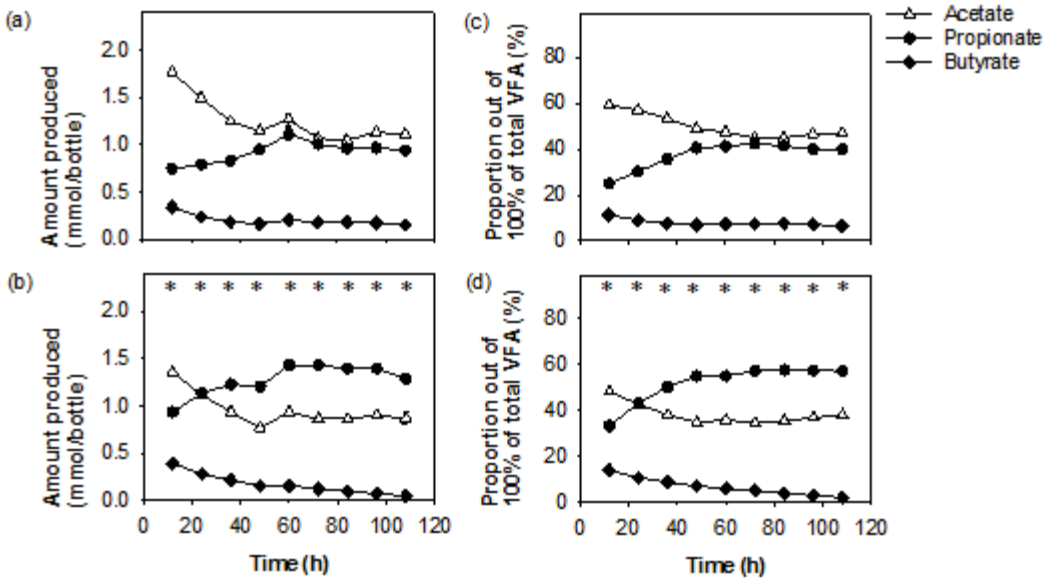

Figure 5.3 Volatile fatty acids (acetate, propionate and butyrate) produced (a; b) and the proportion of each VFA produced (c; d) by sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor (a; c) and in the presence of acetylene (b; d) over time. The * marks the times the serial transfers occurred and the bottles received acetylene. Data are presented as means ± standard errors of the mean (n = 8).

The branched and minor short chain fatty acids isobutyrate, isovalerate, valerate and caproate were also measured. The amount of valerate decreased significantly (p = 0.0001) by 41.7% at 108 h in the presence of acetylene to 0.074 mmol/bottle (Figure 5.4b) as compared to 0.13 mmol/bottle in the incubations with no inhibitor (Figure 5.4a). The amounts of isobutyrate (p = 0.0001), isovalerate (p = 0.0001) and caproate (p = 0.0001) also decreased significantly in the presence of acetylene as compared to incubations with no inhibitor, but the amounts of these branched and minor short-chain fatty acids were very small (< 0.03 mmol/bottle), and sometimes even less than 0.01 mmol/bottle. The total amount of VFA produced after 108 h of inhibition of methane production with acetylene was not significantly affected (p = 1.000) compared to the incubations without inhibitor.

Lactate, formate and ethanol were also measured, but these were detected at very small concentrations (Appendix 2; Table A.2.1). In incubations without acetylene, lactate, formate and ethanol were less than 0.01 mmol/bottle. In the presence of acetylene, lactate was less than 0.01 mmol/bottle, but small amounts of formate were detected that were not more than 0.14 mmol/bottle. Ethanol was also

detected, but was not more than 0.10 mmol/bottle. Therefore, no significant amounts of these three products accumulated and these were not considered further for any calculations.

The mean amounts and proportions of each VFA in the absence of inhibitor, and presence of acetylene during *in vitro* serial transfer over time are provided in Appendix 2 (Table A.2.1).

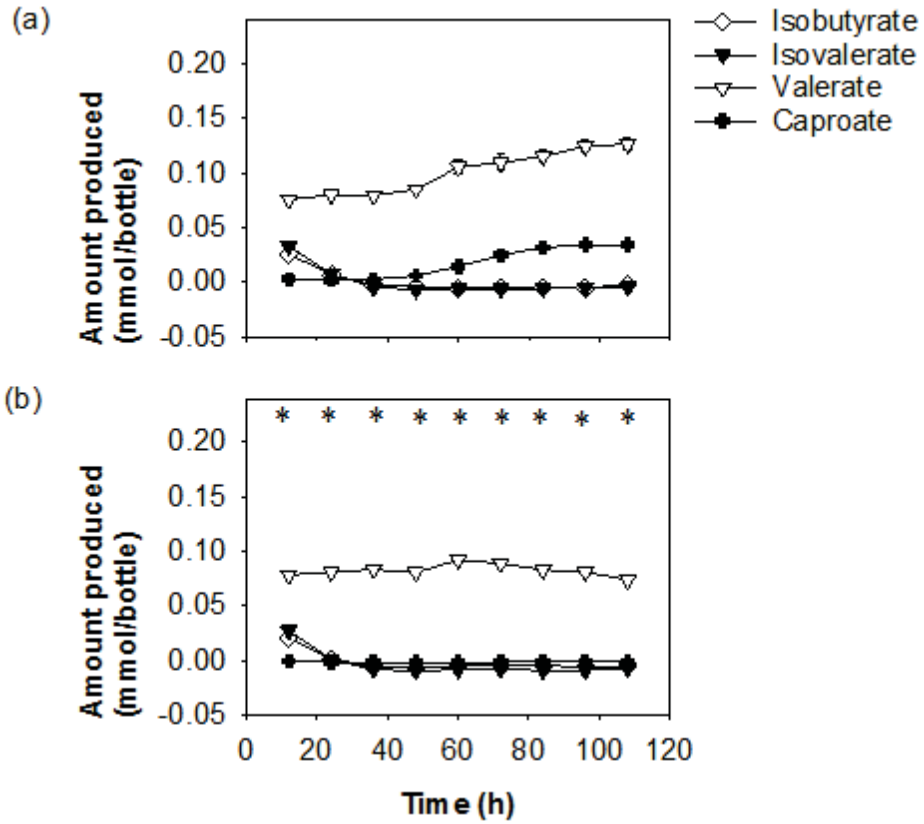

Figure 5.4 Branched and minor short-chain fatty acids (isobutyrate, isovalerate, valerate and caproate) produced by sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor (a) and in the presence of acetylene (b) over time. The * marks the times the serial transfers occurred and the bottles received acetylene. Data are presented as means ± standard errors of the mean (n = 8).

5.4.3 Changes in the microbial community during inhibition of methane production in serial batch *in vitro* fermentation

Total archaeal 16S rRNA gene numbers decreased approximately two-fold in incubations without acetylene (Figure 5.5a), and were significantly lower at 108 h as compared to 12 h ($p = 0.042$). This is completely in agreement with the two-fold

decrease in methane formation after 108 h without acetylene (Figure 5.2a). With the addition of acetylene, total archaeal numbers decreased significantly ($p = 0.0001$) more than 100-fold as compared to the non-inhibited incubations (Figure 5.5b), and this is comparable with the reduction in methane formation by more than 100-fold at 108 h (Figure 5.2b). In contrast, total bacterial numbers were stable ($p = 1.000$) even when methane formation was inhibited using acetylene and were comparable to the incubations with no inhibitor.

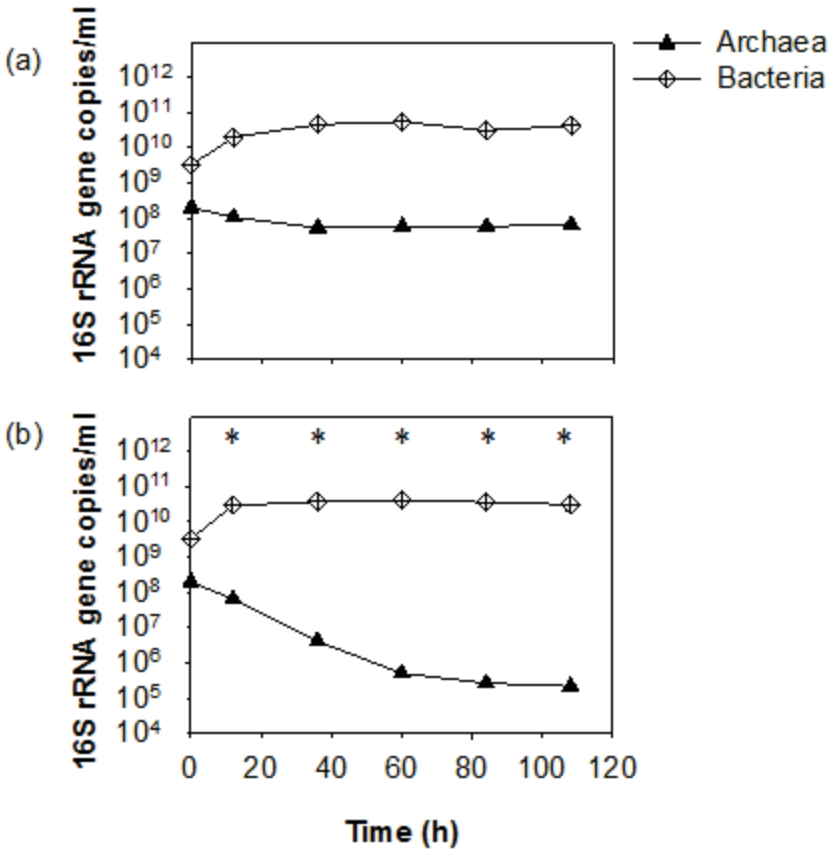

Figure 5.5 Rumen archaeal and bacterial 16S rRNA gene numbers in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor (a) and in the presence of acetylene (b) over time. The * marks the times the serial transfers occurred and the bottles received acetylene. Data are presented as means ± standard errors of the mean, which was smaller than the symbols ($n = 8$). The data from 0 h represents the 16S rRNA numbers in the preparation of rumen fluid contents and Mould's medium used to start the serial transfer experiments.

Changes in archaeal and bacterial community composition were also studied. There were some changes in the archaeal community structure in the non-inhibited series from 36 h (Figure 5.6a), at the same time as there was the two-fold decrease in absolute archaeal numbers (Figure 5.5a). In non-inhibited incubations

at 0 and 12 h, the major archaea were members of the *Methanobrevibacter gottschalkii* clade, normal dominant members of the archaeal community in rumen (Janssen & Kirs 2008). From 36 h onwards, there was a significant decrease (9.09% to 44.6%) in the relative abundance of *Methanobrevibacter gottschalkii* ($p < 0.0001$) and an increase of more than 100-fold in the relative abundance of *Methanomassiliicoccales* Group 9 sp. ISO4-G1 ($p < 0.0001$), and this continued until 84 h. The increase in relative abundance of *Methanomassiliicoccales* Group 9 sp. ISO4-G1 might be due to its potential to grow and compete better than other methanogens in the serial transfer system. Very little is known about these methanogens. From 60 to 108 h, there was also a significant increase in the relative abundance of *Methanomassiliicoccales* Group 12 sp. ISO4-H5 (7.12 to 20.4-fold; $p = 0.024$). The relative abundance of *Methanomassiliicoccales* Group 10 sp. also increased significantly (4.75 to 8.71-fold; $p = 0.021$) from 36 h to 60 h, and then started decreasing ($p = 0.003$) from 84 to 108 h. These changes in the archaeal community, even in the absence of inhibitor, are likely to be due to selection of archaea in the *in vitro* serial batch system, and may also be due to serial transfer every 12 h, rather than the more constant flow through the normal rumen system.

In the presence of acetylene, total archaeal numbers were dramatically reduced (Figure 5.5b) but the remaining archaeal community did not change as much as in the non-inhibited incubations (Figure 5.6b). There was a significant decrease in the relative abundance of members of the *Methanobrevibacter gottschalkii* clade (11.8% to 34.7%; $p < 0.002$), and an increase in relative abundance of *Methanosphaera* sp. Group 5 (1 to 3-fold; $p = 0.022$) from 60 h onwards compared to non-inhibited incubations at 12 h. The relative abundance of *Methanosphaera* sp. ISO3-F5 also showed changes over time, increasing significantly (3.8-fold; $p = 0.0001$) until 84 h, and then at 108 h there was trend towards an increase (< 1-fold $p = 0.078$) compared to non-inhibited incubations. At 108 h, the relative abundance of members of the *Methanobrevibacter gottschalkii* clade started increasing again by 30.2%.

In contrast to the stability in absolute bacterial numbers (Figure 5.5a and 5.5b), the bacterial community composition appeared to be strongly affected in both the non-inhibited and acetylene-treated bottles during serial batch transfer (Figure 5.7a and 5.7b). The seven most abundant rumen bacterial groups globally are from

the genera *Prevotella*, *Butyrivibrio* and *Ruminococcus,* unclassified members of the families *Lachnospiraceae* and *Ruminococcaceae*, and unclassified members of the orders *Bacteroidales* and *Clostridiales* (Henderson *et al.* 2015). These were also observed during *in vitro* serial batch transfer, as these are major the fermentative bacteria that ferment feed to volatile fatty acids in the rumen. There was first significant increase by 53.1% at 12 h and then a decrease by 18.5% from 36 h in the relative abundance of *Prevotella* spp. ($p < 0.0001$), and an increase in the relative abundance of *Streptococcus* spp. by more than 100-fold ($p = 0.010$) and unclassified members of the family *Lachnospiraceae* (approximately 30%; $p < 0.0001$) over time in the non-inhibited incubations (Figure 5.7a). The relative abundance of unclassified members of the order *Bacteroidales* increased (1.3-fold; $p = 0.008$) and *Clostridiales* decreased (65.4%; $p < 0.0001$) significantly after 108 h. The relative abundance of unclassified members of the family *Ruminococcaceae* ($p < 0.0001$) and *Veillonellaceae* ($p < 0.0001$) decreased by 63.7% and 90.0% respectively, whereas *Pseudobutyrivibrio* spp. increased significantly (10.4-fold; $p < 0.0001$) in non-inhibited incubations over time. In the presence of acetylene, there was first a trend towards increase by 73.5%, and then a significant decrease by 34.2% in the relative abundance of *Prevotella* spp. ($p < 0.0001$) at 108 h, while the relative abundance of unclassified members of the family *Lachnospiraceae* decreased significantly (75.4%; $p < 0.0001$) after 108 h compared to non-inhibited incubations (Figure 5.7b). The relative abundance of *Streptococcus* spp. increased significantly (3.3-fold; $p = 0.0001$) and *Fibrobacter* spp. decreased significantly (59.8%; $p = 0.003$) at 108 h compared to non-inhibited incubations. The relative abundance of unclassified members of the order*Clostridiales* ($p = 0.0001$) decreased over time by 62.8% in the acetylene treatment, whereas the relative abundance of unclassified members of the family *Ruminococcaceae* ($p < 0.0001$) and *Veillonellaceae* ($p < 0.0001$) decreased by 63.6% and 50.1% respectively, while *Pseudobutyrivibrio* spp. decreased significantly (43.4%; $p < 0.0001$) in the presence of acetylene at 108 h compared to non-inhibited incubations at 108 h. Therefore, the bacterial community seemed to change both in response to the serial batch system and to the presence of acetylene.

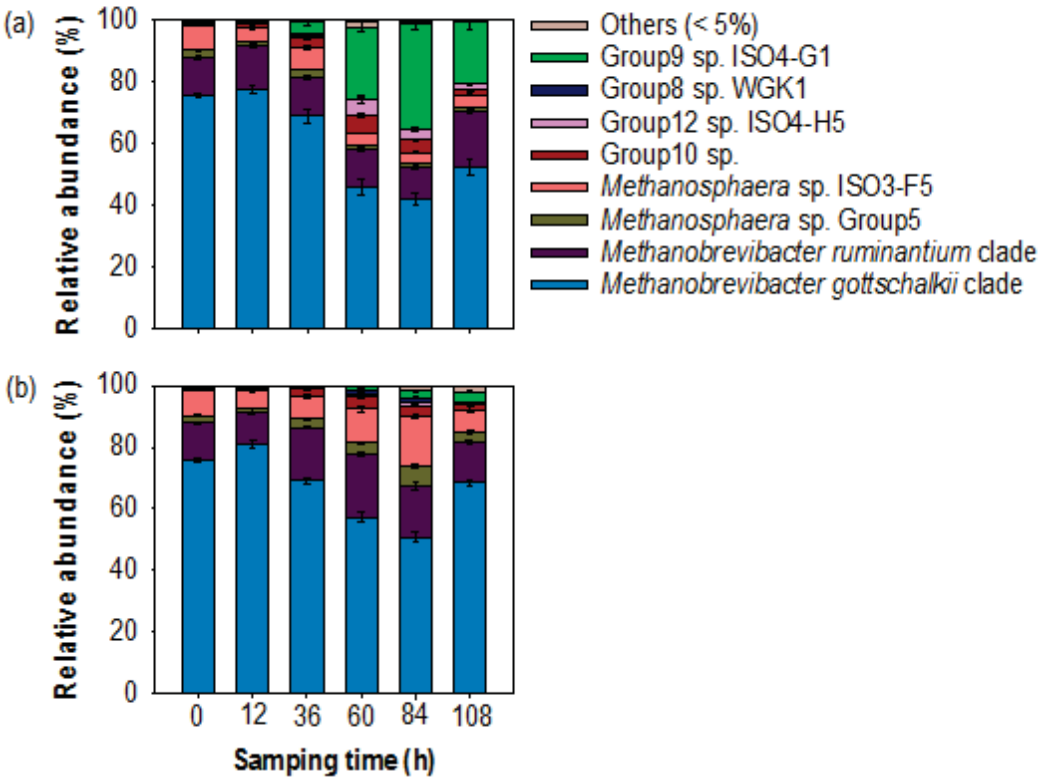

Figure 5.6 Archaeal community composition in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor (a) and in the presence of acetylene (b) over time. Data are presented as means ± standard errors of the mean (n = 8). Archaeal 16S rRNA gene sequences were classified at the species level. Group 8, 9, 10 and 12 sp. belong to the order *Methanomassiliicoccales*. The data from 0 h represents the archaeal community composition in the preparation of rumen fluid contents and Mould's medium used to start the serial transfer experiments. All taxa present at < 5% in all samples are grouped together as "Others" to simplify the figure.

The microbial community that formed in the serial batch transfer system was not really comparable with that in the rumen and does not represent likely changes in microbial community that could happen in the absence of methanogens in the rumen. There was very little lactate detected in the fermentations, but a large population of *Streptococcus* spp. developed in the acetylene-amended series. *Streptococcus* spp. are known to form lactate (Russell & Robinson 1984, Russell & Hino 1985, Owens *et al.* 1998), but there must have been other bacteria present that ferment lactate to acetate and propionate. This would be consistent with the shift to more propionate formation observed when methane formation was inhibited with acetylene (Figure 5.3b). Members of the order *Bacteroidales*, members of which are known to produce propionate, acetate and succinate from lactate (Macy *et al.* 1978, Schultz & Breznak 1979), and *Succiniclasticum* spp., a ruminal bacterium

that converts succinate to propionate (van Gylswyk 1995), were present in both the non-inhibited and acetylene-treated serial transfers. In the serial transfers in the presence of acetylene, the relative abundance of *Succiniclasticum* spp. increased to approximately 4% by 36 h and then started decreasing to approximately less than 2% at 108h. In the non-inhibited serial transfers it was less than 1.5% at 12 h and at the end of the experiment it had increased to 2.3% of the total 16S rRNA genes.

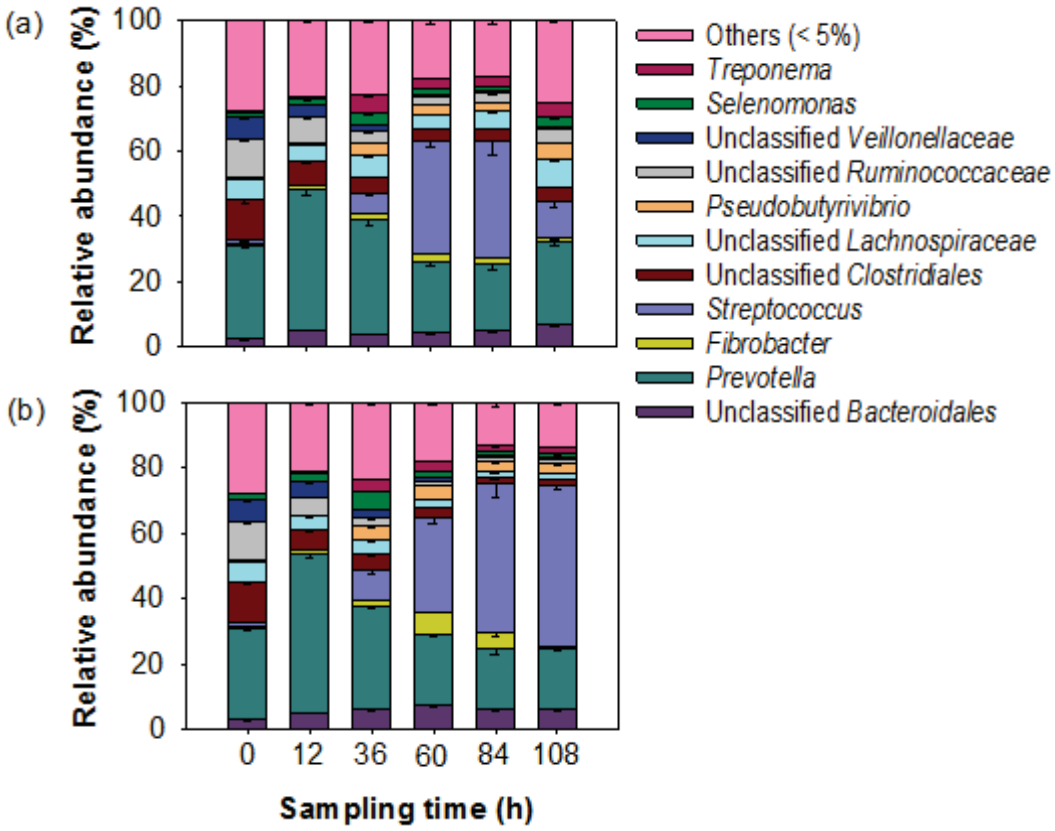

Figure 5.7 Bacterial community composition in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor (a) and in the presence of acetylene (b) over time. Data are presented as means ± standard errors of the mean (n = 8). Bacterial 16S rRNA gene sequences were classified at the genus level, except the groups containing sequences not able to be classified down to the genus level, which were represented as "unclassified" within the lowest ranked named taxon to which they belonged (family or order). The data from 0 h represents the bacterial community composition in the preparation of rumen fluid contents and Mould's medium used to start the serial transfer experiments. All genera present at < 5% in all samples are grouped together as "Others" to simplify the figure.

Principal component analysis (PCA) of bacterial community structure in both non-inhibited and acetylene-treated bottles over time during *in vitro* serial

batch fermentation showed that there were differences over time and by treatment (Figure 5.8a). Bacterial communities in non-inhibited incubations clustered together and then, from 36 h onwards, they clustered apart as compared to 12 h (Figure 5.8b). In acetylene-treated incubations, the samples clustered together and were close to samples in non-inhibited incubations at 12 h (Figure 5.8a). However, the samples in acetylene-treated incubations also clustered apart from 36 h onwards as compared to 12 h (Figure 5.8c). The clustering of samples in non-inhibited and acetylene-treated incubations were different from each other suggesting an additional effect of acetylene.

The most abundant and prevalent genus-level groups of bacteria in the absence of inhibitor and in the presence of acetylene during the *in vitro* serial transfer experiment are listed in Appendix 2 (Table A.2.3 and A.2.4 respectively).

5.4.4 Measurement of homoacetogenesis using short-term *in vitro* assay

Homoacetogenesis was measured using the short-term (8 h) *in vitro* homoacetogenesis assay at the time the rumen contents were sampled from the sheep, and after 108 h of serial transfer in the presence and absence of acetylene. The short-term assay employed further additional controls in these short-term assays. BES was added to inhibit residual methanogenesis to determine if homoacetogenic activity could increase, and chloroform was used as a partial inhibitor of homoacetogens. Using this assay, it was possible to assess changes in the amount of homoacetogenic activity (including homobutyrogenesis) after sheep rumen contents had been exposed to acetylene for 108 h, with serial transfer of the incubations to allow homoacetogens to grow. The original dilution of rumen contents contained 20% rumen contents, and after 9 transfers using 33% inoculum each 12 h, only 9.3×10^{-6} of the original contents would remain in the short-term assay. This means that any homoacetogenesis would have to be attributed to growing cells.

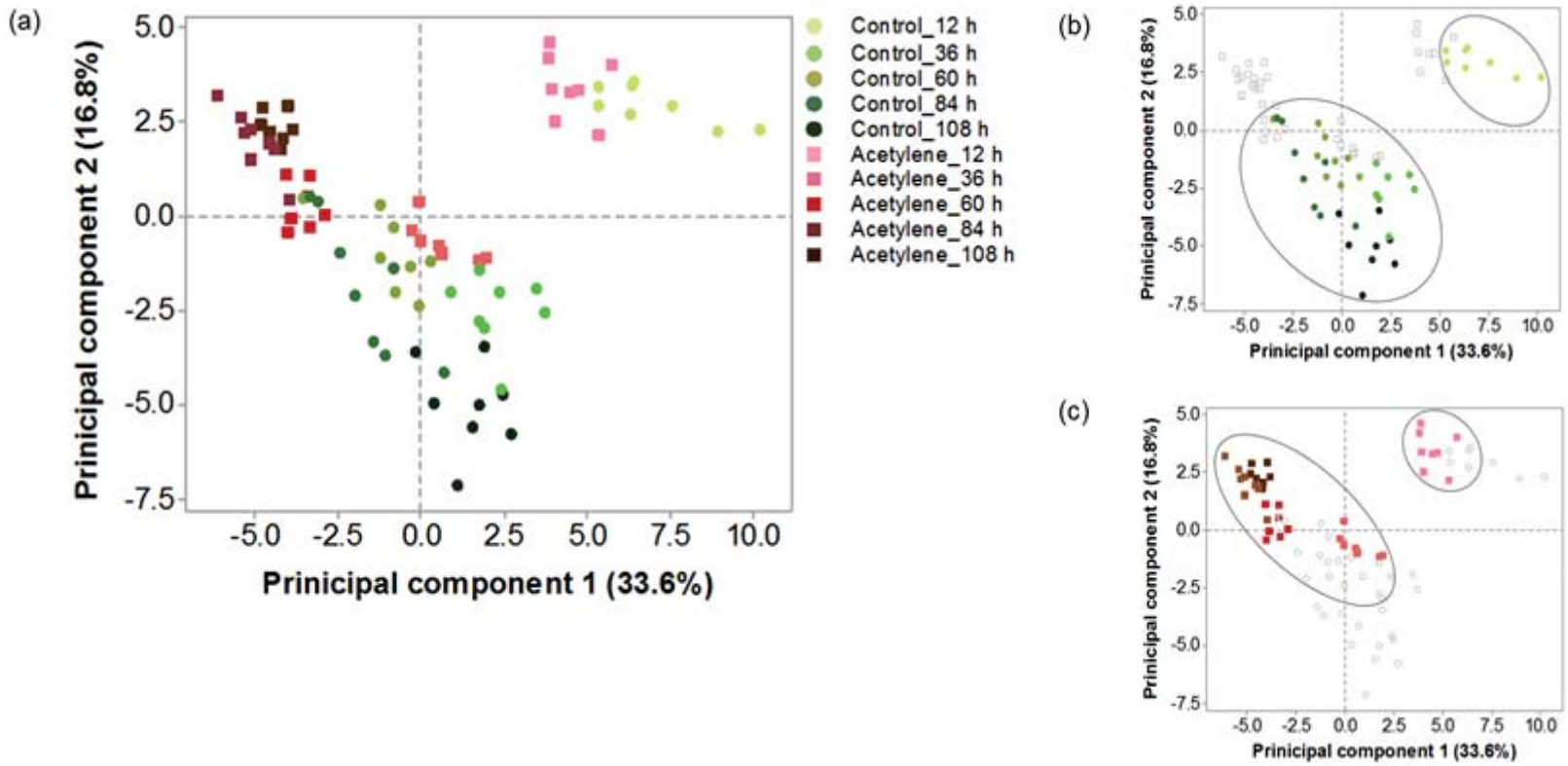

Figure 5.8 Scatter plot of principal component analysis (PCA) of bacterial communities in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor and in the presence of acetylene (a) over time. Data points highlighted in (b) represent bacterial communities in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor and (c) in the presence of acetylene over time. The amounts of variation explained by principal component 1 and 2 are 33.6% and 16.8%% respectively. Panels (a), (b) and (c) show the same data on the same axes. Open grey symbols present points not being considered in any one plot.

5.4.4.1 Methane decreased with addition of inhibitors

Fresh rumen contents from sheep formed methane in the short-term (8 h) assays (Figure 5.9a). BES and chloroform inhibited this methane production significantly by 90.4% ($p < 0.0001$) and 97.2% ($p < 0.0001$) respectively. The concentrations of BES and chloroform were lower than in the original short-term assay described in Chapter 4, but very similar results were obtained. More hydrogen was also produced in the BES (5.84% v/v of total gas; $p < 0.0001$) and chloroform treatments (5.37% v/v of total gas; $p < 0.0001$) than in the assays with no inhibitor (0.28% v/v of total gas). The amount of total gas produced was also significantly affected by addition of BES ($p = 0.026$) decreasing it by 12.6%, and there was a trend towards a decrease of 7.89% caused by the addition of chloroform ($p = 0.086$) as compared to assays with no inhibitor.

After nine transfers of rumen contents in the serial batch system in the absence of an inhibitor, addition of BES and chloroform reduced methane production significantly by 94.2% ($p < 0.0001$) and 96.3% ($p < 0.0001$) respectively, compared to assays with no inhibitor (Figure 5.9b). This was very similar to their effects in the short-term assays when fresh rumen contents were used. More hydrogen was formed and increased significantly by 9- to 10-fold, making up 6.21% ($p = 0.002$) and 6.82% ($p = 0.002$) v/v of total gas, when BES and chloroform were added, respectively, as compared to assays with no inhibitor, where hydrogen made up 0.62% v/v of the total gas. The amount of total gas produced seemed to be significantly unaffected over BES ($p = 0.358$) or chloroform ($p = 0.999$) addition as compared to assays with no inhibitor.

When the acetylene-treated inoculum, after nine transfers in the serial batch system, was used in the short-term assay, virtually no methane was formed even in the absence of BES and chloroform, indicating that very few active methanogens were present (Figure 5.9c), although some carryover of acetylene could not be ruled out. A small amount of hydrogen was detected in assays with no inhibitor (0.94% v/v of total gas), BES (1.36% v/v of total gas) and chloroform (1.94% v/v of total gas) treatments (Figure 5.9c). The amounts of hydrogen formed by the acetylene-treated inoculum incubated in the presence of BES or chloroform was significantly lower by approximately 60 to 80% ($p = 0.021$) than when naïve rumen

contents (Figure 5.9a) or serially transferred contents that had not been exposed to an inhibitor (Figure 5.9b) were treated with BES or chloroform ($p = 0.010$). This suggests that fermentation pathways had changed in the acetylene-treated inoculum compared to the serially-transferred inoculum that was not exposed to acetylene, or compared to the initial rumen contents. There appears to have been a shift away from hydrogen production, or alternative hydrogen utilisers had become (more) active.

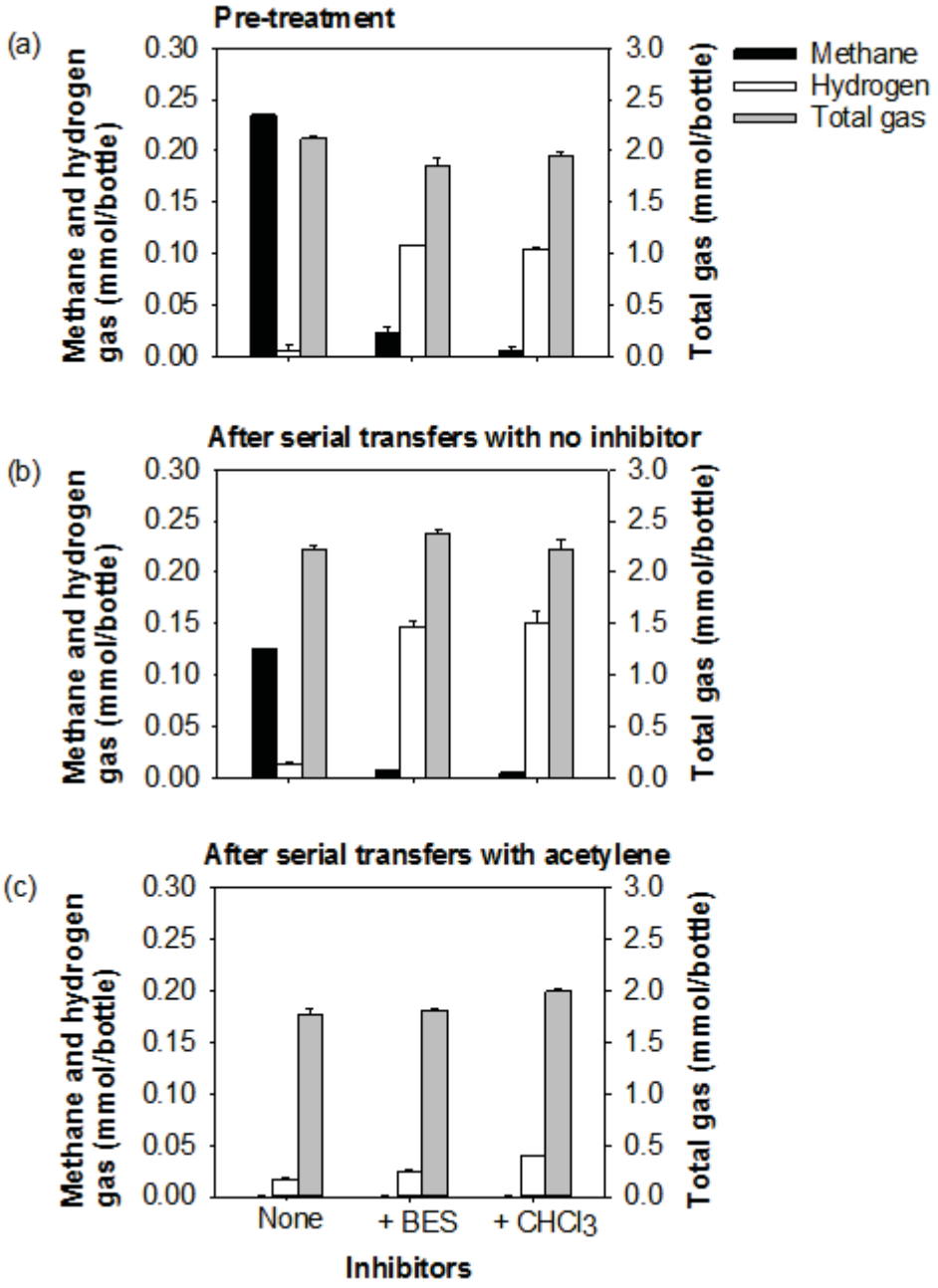

Figure 5.9 Methane, hydrogen and total gas produced *in vitro* over 8 hours by fresh sheep rumen contents (a) and after nine serial transfers without an inhibitor (b) or after nine serial transfers in the presence of acetylene (c). Each inoculum was tested in the short-term assay in the absence of inhibitor and in the presence of BES or chloroform. Data are presented as means ± standard errors of the mean ($n = 2$).

5.4.4.2 Changes in volatile fatty acids formed

Differences in the amount of the different VFA produced were also observed in the short-term incubations conducted with the original rumen contents and after nine serial transfers. When the original rumen contents were treated with BES in the short-term assay, the amount of acetate decreased significantly ($p < 0.0001$) by 28.9%, whereas propionate increased significantly ($p = 0.023$) by10.2%, and there was no significant effect on butyrate ($p = 0.726$), compared to the assays with no inhibitor (Figure 5.10a). With addition of chloroform to the short-term assay, the effects were similar: acetate decreased ($p < 0.0001$) by 29.6%, propionate increased significantly ($p = 0.004$) by 18.3% and there was no significant effect on butyrate ($p = 0.117$) compared to assays with no inhibitor.

After nine serial transfers without addition of acetylene (Figure 5.10b), the amount of acetate decreased significantly ($p = 0.001$) by 40.7% and butyrate decreased significantly ($p < 0.0001$) by 44.8%, and propionate formed in the short-term assays increased significantly ($p = 0.005$) by 52.8% as compared to the short term assays made using fresh contents (Figure 5.10a). The VFA formed did not change as much with further addition of BES and chloroform to the short-term assay as they did with fresh rumen contents. This might be explained by the changes in the bacterial community over the 108 h of serial transfer (Figure 5.7a), as the relative abundances of *Prevotella* spp., unclassified members of the families *Veillonellaceae* and *Ruminococcaceae*, and the order *Clostridiales*, which were higher at the beginning (12 h), decreased after nine serial transfers by 108 h. There were also shift in the amounts of the different VFA formed even in the absence of inhibitor over serial transfer, with more propionate and less butyrate after 108 h compared to after 12 h (Figure 5.3). The resultant community appeared to have only a small ability to change to produce less acetate and more propionate when BES and chloroform were added (Figure 5.10c).

When the acetylene-treated inoculum was used in the short-term assay (Figure 5.10c), significantly less acetate (34.0 to 50.5%; $p < 0.0001$) and butyrate (62.7 to 96.4%; $p < 0.0001$) and more propionate (20.7 to 87.1%; $p < 0.0001$) were produced compared to fresh rumen contents and parallel serially-transferred inoculum without acetylene (Figure 5.10a and 5.10b). Addition of inhibitors (BES or chloroform) to the short-term assay had no significant effects on the VFA,

resulting in no significant change in acetate ($p > 0.300$) and propionate ($p > 0.300$) production, compared to when the acetylene-treated inoculum was incubated in assays without inhibitor. This is probably also linked to changes in bacterial community structure over the period of inhibition of methane production with acetylene during serial transfer (Figure 5.7b). It appears that the community that developed in the presence of acetylene in the serially-transferred batch system was already functioning in the absence of methanogens, and so adding BES or chloroform had very little impact on the major fermentation pathways.

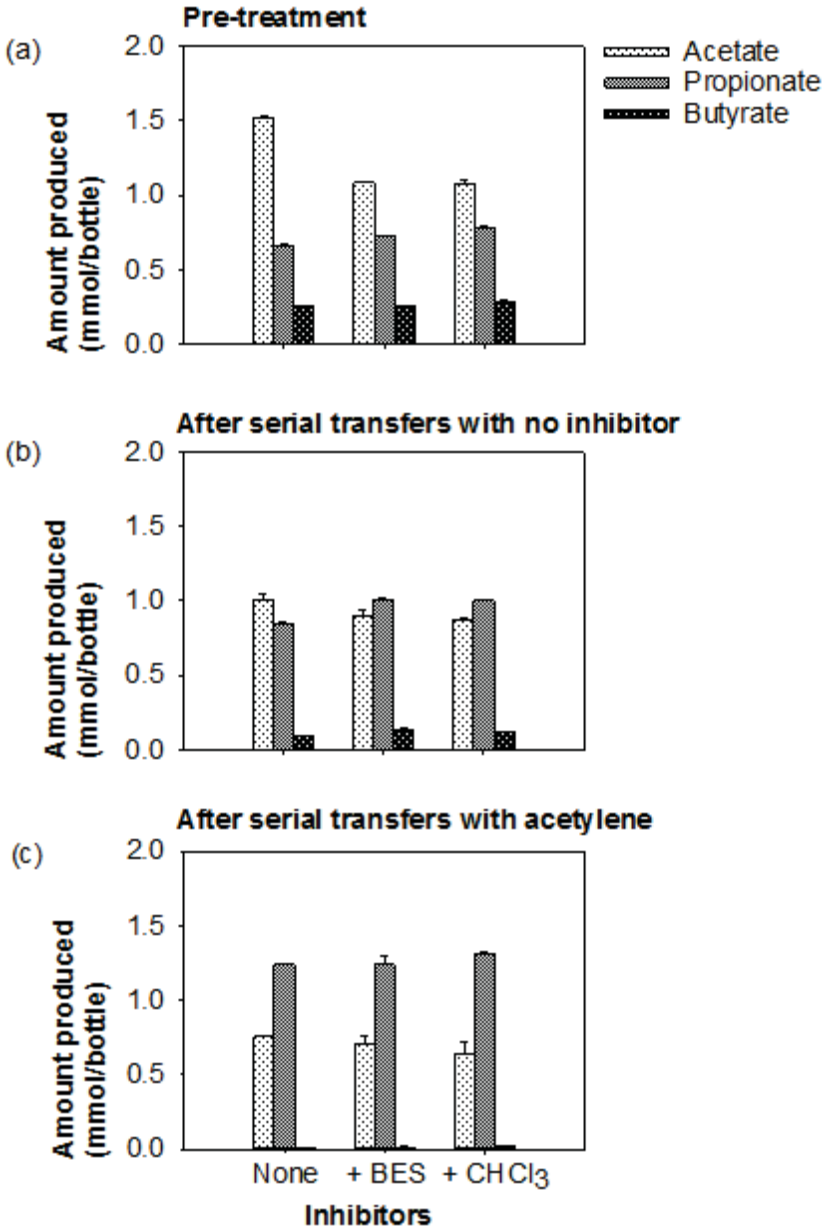

Figure 5.10 Volatile fatty acids (acetate, propionate and butyrate) produced *in vitro* over 8 hours by fresh sheep rumen contents (a) and after nine serial transfers without an inhibitor (b) or after nine serial transfers in the presence of acetylene (c). Each inoculum was tested in the short-term assay in the absence of inhibitor and in the presence of BES or chloroform. Data are presented as means ± standard errors of the mean ($n = 2$).

The *in vitro* short-term assay results seem to be consistent with proportions of acetate, propionate and butyrate produced in the serial transfer bottles with and without inhibitor. In both systems, there was a decrease in acetate and butyrate, and increase in propionate formation by rumen contents that had been exposed to acetylene for 108 h and which were no longer producing methane (Figure 5.3).

5.4.4.3 Homoacetogens were more active when methane formation was inhibited with acetylene in serial batch fermentation

Homoacetogenesis was measured using the 8-h homoacetogenesis assay with fresh rumen contents and after nine serial transfers of the rumen contents without and with acetylene. Homobutyrogenesis, the formation of butyrate from CO_2 and H_2, was also measured. The quantity of ^{13}C-labelled acetate produced via homoacetogenesis ($^{13}A_{ha}$) and ^{13}C-butyrate produced via homobutyrogenesis ($^{13}B_{hb}$) were calculated using data described in the Appendix 2 (Table A.2.5), and corrected for VFA inter-conversion by using fractional amounts of VFA inter-conversion (equations 1 and 2) described in detail in Chapter 4 and Figure 4.14.

$$^{13}A_{ha} = {}^{13}A - f_{pa} \times {}^{13}P - f_{ba} \times {}^{13}B + f_{ap} \times {}^{13}A + f_{ab} \times {}^{13}A \quad (1)$$

$$^{13}B_{hb} = {}^{13}B - f_{pb} \times {}^{13}P - f_{ab} \times {}^{13}A + f_{bp} \times {}^{13}B + f_{ba} \times {}^{13}B \quad (2)$$

Finally, from the ratio excess of $^{13}CO_2$ measured using IRMS ($r^{13}CO_2$), the total quantity of acetate (unlabelled and labelled) produced via homoacetogenesis (A_{ha}), and the total quantity of butyrate (unlabelled and labelled) produced via homobutyrogenesis (B_{hb}) were calculated (equations 3 and 4).

$$A_{ha} = {}^{13}A_{ha}/r^{13}CO_2 \quad (3)$$

$$B_{hb} = {}^{13}B_{hb}/r^{13}CO_2 \quad (4)$$

The amount of acetate formed from homoacetogenesis and butyrate from homobutyrogenesis was expressed as both the amount produced in µmol/bottle (Figure 5.11), and as a percentage of total acetate ($A_{ha}/A \times 100$) and butyrate produced ($B_{ha}/B \times 100$; Figure 5.12).

Using fresh rumen contents, the amount of acetate formed from homoacetogenesis was nearly 16.8 µmol/bottle and butyrate from

homobutyrogenesis was 2.49 µmol/bottle in the short-term assays without inhibitor (Figure 5.11a). Addition of BES to the short-term assay resulted in significantly increased acetate formation from homoacetogenesis ($p = 0.004$) by 62.5% to 27.3 µmol/bottle, and a significant decrease in butyrate formation from homobutyrogenesis ($p = 0.004$) by 60.1% to 0.97 µmol/bottle as compared to assays with no inhibitor. Addition of chloroform reduced acetate formation from homoacetogenesis by 60.3% and butyrate from homobutyrogenesis by 60.5%, to 3.26 µmol/bottle ($p = 0.002$) and 0.99 µmol/bottle ($p = 0.002$) respectively and significantly, as compared to assays without inhibitor. This is in agreement with the results from Chapter 4. Inhibiting methanogens allowed an increase in homoacetogenesis, but chloroform, which inhibits homoacetogens as well as methanogens, reduces homoacetogenesis.

After nine serial transfers of rumen contents in the absence of acetylene (Figure 5.11b), the amount of acetate formed from homoacetogenesis was nearly 8.77 µmol/bottle regardless of whether BES or no inhibitor was added to the assay ($p = 0.827$), whereas butyrate from homobutyrogenesis was 0.23 µmol/bottle in assays without inhibitor and increased significantly ($p < 0.0001$) by 1.85-fold to 0.66 µmol/bottle in the presence of BES. Homoacetogenesis was therefore reduced to nearly half of what it was in the fresh rumen contents ($p = 0.005$). Chloroform addition to the short-term assay significantly reduced acetate formation from homoacetogenesis even further ($p = 0.003$) by 74.7% to 2.23 µmol/bottle, and significantly increased butyrate from homobutyrogenesis ($p = 0.001$) by 1.41-fold to 0.56 µmol/bottle as compared to assays with no inhibitor.

In short-term assays using the acetylene-treated inoculum (Figure 5.11c), the amount of acetate formed from homoacetogenesis was 16.2 µmol/bottle and butyrate formed from homobutyrogenesis was very small or undetectable in assays without inhibitor. Thus, acetylene treatment had almost doubled homoacetogenesis compared to the parallel serial transfer series without acetylene. Addition of BES did not increase the amount of acetate from homoacetogenesis; indeed, it reduced it somewhat by 27.2% to 11.8 µmol/bottle ($p = 0.045$), and butyrate formation from homobutyrogenesis was still undetectable. With the addition of chloroform, acetate formed from homoacetogenesis was almost completely abolished by 96.9%, being reduced to 0.50 µmol/bottle ($p = 0.001$), and butyrate formed from

homobutyrogenesis was very small, 0.0085 μmol/bottle, compared to assays with no inhibitor.

The results confirmed the occurrence of homoacetogenesis in fresh sheep rumen fluid incubated *in vitro* as also confirmed in Chapter 4. After 108 h of inhibition of methane production by serial transfer, homoacetogenic activity decreased compared to fresh rumen fluid. Inhibition of methanogenesis in the serial batch transfer system by the addition of acetylene resulted in twice the amount of homoacetogenesis compared to the parallel transfers without acetylene. This showed that, in the absence of methanogenesis, homoacetogenic activity had the potential to increase. After nine transfers using 33% inoculum each 12 h, only 9.3×10^{-6} of the original rumen contents would be present in the 8-h assay. Since the serial batch transfer resulted in an almost 100,000-fold dilution, the homoacetogens must have been able to grow, and apparently were more competitive in the absence of methanogens.

5.4.4.4 Hydrogen concentration versus relative rate of hydrogen metabolism for homoacetogens

The theoretical relative rate of hydrogen metabolism by homoacetogens versus the concentration of hydrogen was calculated as described in Chapter 4 (equation 21). In fresh rumen contents incubated without inhibitor for 12 h, the hydrogen concentration was only 0.73 μM and the calculated relative rate of metabolism of homoacetogens was virtually zero (Figure 5.13b). After 108 h of serial transfers with no inhibitor, the hydrogen concentration increased by 3.03-fold to 2.94 μM, and the calculated relative rate of metabolism of homoacetogens was still small (< 0.004) compared to fresh rumen contents. This is consistent with the small amount of acetate formed from homoacetogenesis after serial transfers with no inhibitor (Figure 5.11b). When the serial batch transfers were made in the presence of acetylene, the hydrogen concentration increased by 56.5-fold to 42.0 μM after 12 h and the calculated relative rate of metabolism of homoacetogens also increased to 0.055, compared to fresh rumen contents. However, after 108 h of inhibition of methane production in the presence of acetylene during serial transfer, the hydrogen concentration decreased by 27.6%, to 30.4 μM and so the calculated relative rate of metabolism for homoacetogens decreased to 0.041 (Figure 5.13c) as compared to

rumen contents at 12 h treated with acetylene. This increased theoretical rate of homoacetogenesis is consistent with the increase in acetate formed from homoacetogenesis after nine serial transfers with acetylene compared to the serial transfers without acetylene (Figure 5.11c). The calculated concentrations of dissolved hydrogen were high enough to allow homoacetogens to be active.

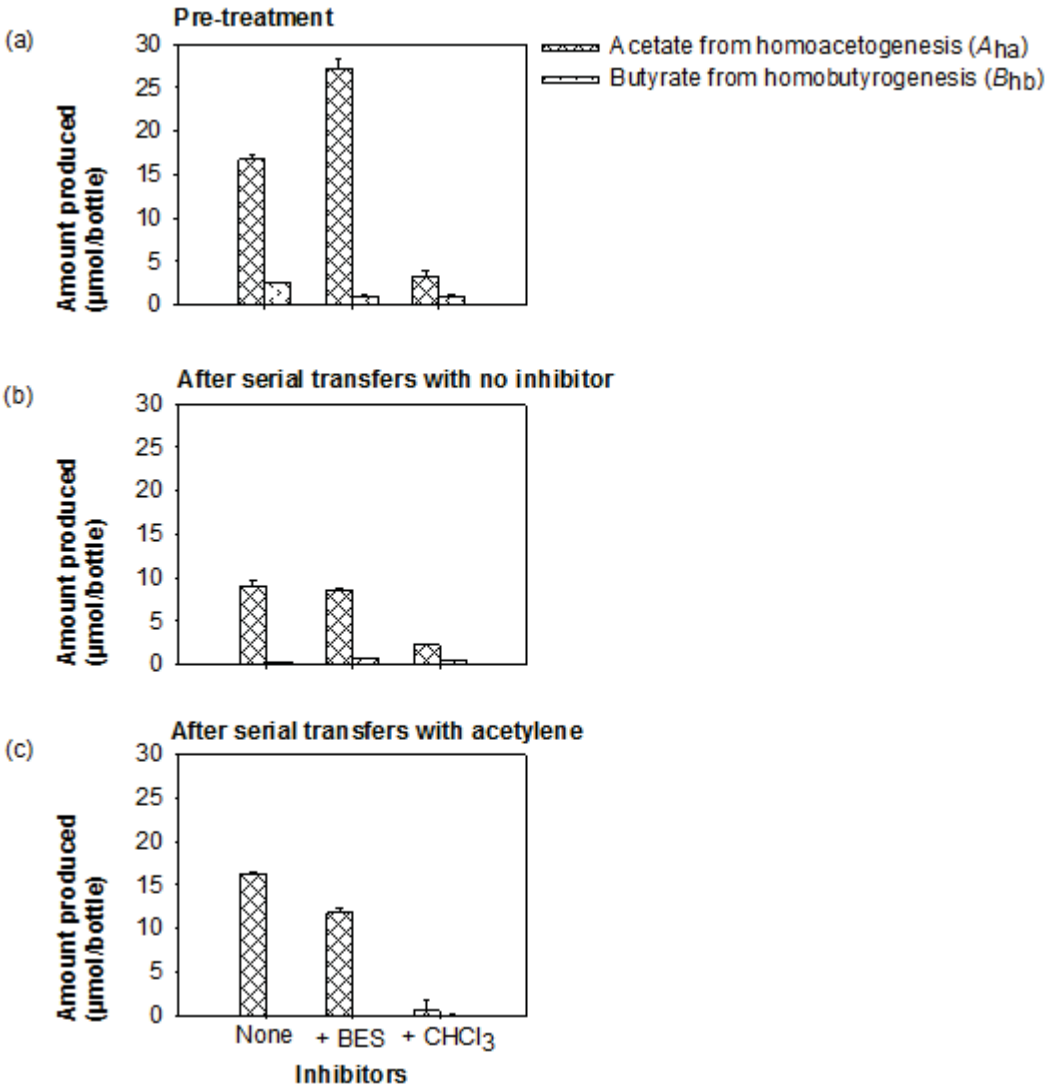

Figure 5.11 Amount of acetate formed from homoacetogenesis (A_{ha}) and butyrate formed from homobutyrogenesis (B_{hb}) *in vitro* over 8 hours by fresh sheep rumen contents (a) and after nine serial transfers without an inhibitor (b) or after nine serial transfers in the presence of acetylene (c). Each inoculum was tested in the short-term assay in the absence of inhibitor and in the presence of BES or chloroform. Data are presented as means ± standard errors of the mean (n = 2).

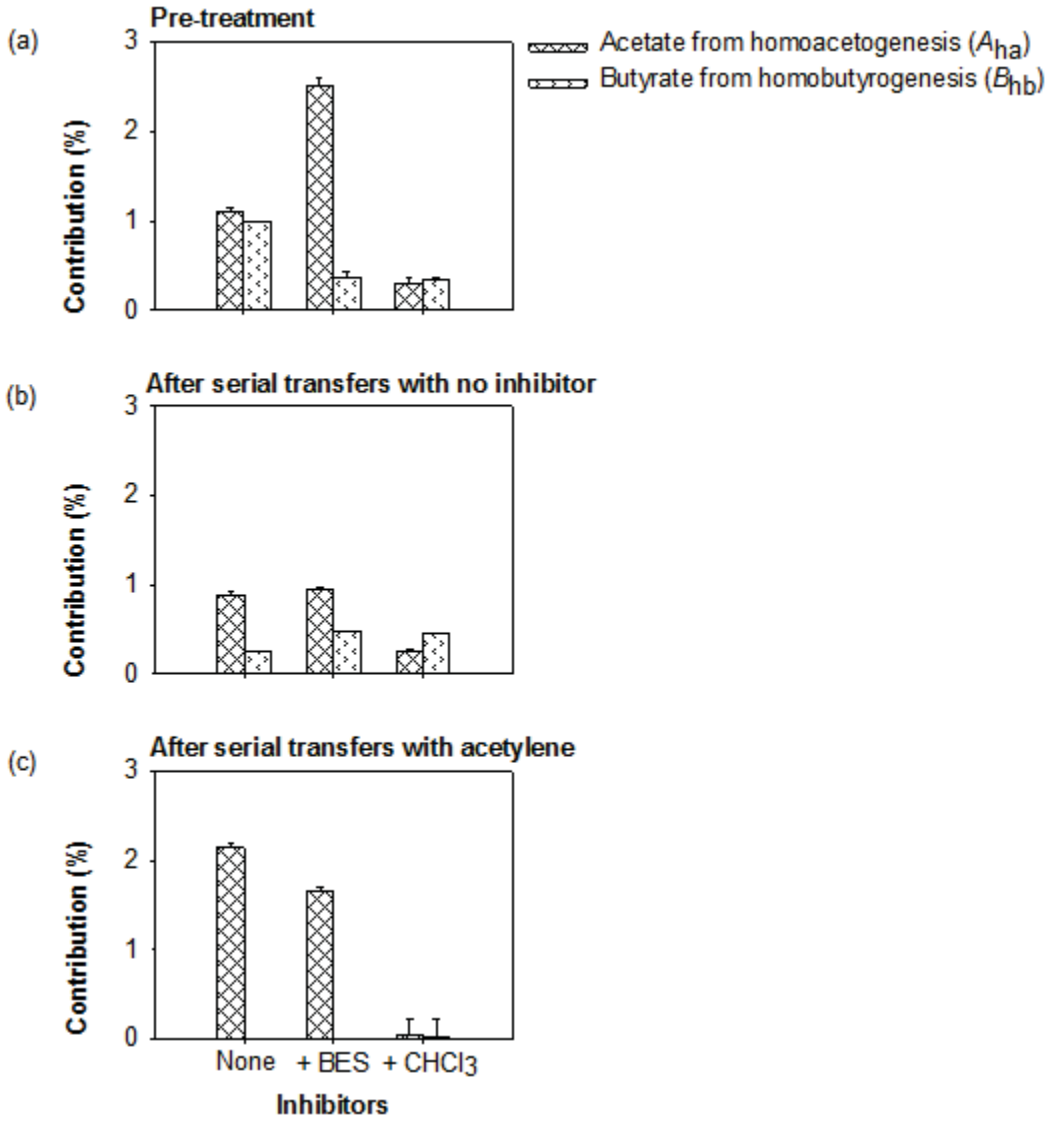

Figure 5.12 Contribution of homoacetogenesis (A_{ha}) and homobutyrogenesis (B_{hb}) to total acetate and butyrate formation *in vitro* over 8 hours by fresh sheep rumen contents (a) and after nine serial transfers without an inhibitor (b) or after nine serial transfers in the presence of acetylene (c). Each inoculum was tested in the short-term assay in the absence of inhibitor and in the presence of BES or chloroform. Data are presented as means ± standard errors of the mean (n = 2).

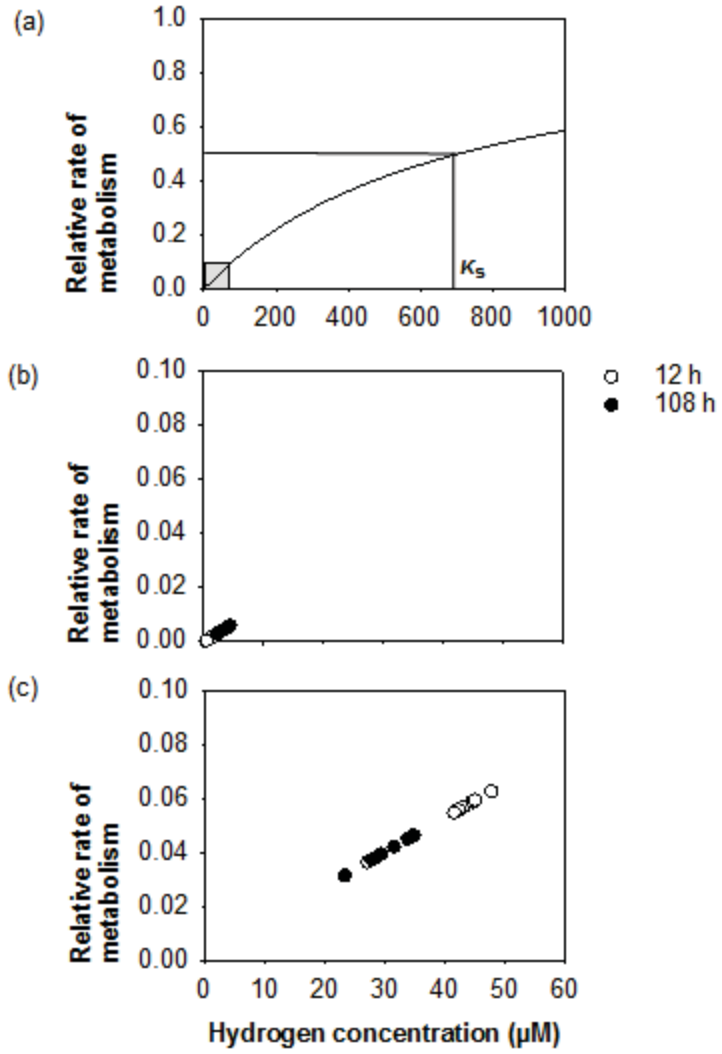

Figure 5.13 Theoretical relative rates of metabolism versus hydrogen concentration for homoacetogens (a) with K_s = 710 µM and S_{min} = 383 nM. The shaded area in (a) was then plotted using actual data points, i.e., dissolved hydrogen concentration versus calculated relative rate of metabolism of homoacetogens in the *in vitro* serial batch fermentation in the absence of inhibitor (b) and in the presence of acetylene (c) over time. Data points from all experimental replicates (n = 8) are plotted, and data from 12 h (white circles) and 108 h (black circles) are shown.

5.4.4.5 Electron balances during short-term *in vitro* rumen fermentation

The electron balance was calculated from the production and utilisation of electrons using following equations, described in detail in Chapter 4:

$$2H \text{ produced} = 2A_f + P_f + 4B_f + 3V_f \quad (5)$$

$$2H \text{ utilised} = 2P_f + 2B_f + 4V_f + 4A_{ha} + 10B_{hb} + 4M + H_2 \quad (6)$$

5.4.4.5.1 Electron utilisation by homoacetogenesis and other products in 8-h assay

Electron utilisation was calculated for each product as a proportion of total electrons utilised in the 8-h assay. The percentages of *2H* utilised in the short-term assay inoculated with fresh rumen fluid (pre-treatment; Figure 5.14a) were 30.3% by methane formation, 0.19% in hydrogen, 42.7% in propionate, 16.2% in butyrate, 7.67% in valerate, 2.16% in homoacetogenesis and 0.80% in homobutyrogenesis. With the addition of BES, *2H* utilisation by methanogenesis dropped to 3.57% of the total *2H* utilised, and the electron flow increased to other products: hydrogen to 4.28%, propionate to 57.5%, butyrate to 20.5%, valerate to 9.51%, and homoacetogenesis to 4.31%, while the electron flow to homobutyrogenesis decreased to 0.38%. In the presence of chloroform, *2H* utilisation for methanogenesis decreased to 1.03%, homoacetogenesis to 0.51% and homobutyrogenesis to 0.39% as compared to assays with no inhibitor. With chloroform addition, *2H* utilisation for hydrogen, propionate, butyrate and valerate formation increased and was comparable to the addition of BES. Therefore, during short-term inhibition of methanogenesis by fresh rumen contents using BES, the *2H* utilisation by homoacetogenesis increased from 2.16% to 4.31%.

When the rumen contents that had been serially transferred with no acetylene for 108 h was used as the inoculum in the 8-h assay (Figure 5.14b), *2H* utilised in methanogenesis was 17.3% of the total *2H* utilisation, hydrogen 0.48%, propionate 58.1%, butyrate 6.51%, valerate 16.3%, homoacetogenesis 1.24% and homobutyrogenesis 0.08%. This drop in *2H* utilisation by methanogenesis in the short-term assay compared to the fresh rumen contents is comparable to the decrease in methane production from the actual serially-diluted transfers (Figure 5.9b). With addition of BES to the short-term assay, the amount of *2H* utilisation by methanogenesis dropped further to only 0.98% and that by hydrogen formation increased to 5.03%, propionate to 69.0%, butyrate to 9.48%, valerate to 14.10%, whereas homoacetogenesis remained the same at 1.18% and homobutyrogenesis used 0.23% of the electrons. In the presence of chloroform, *2H* utilisation for methanogenesis decreased to 0.66%, homoacetogenesis to 0.32% and homobutyrogenesis to 0.20% as compared to the 8-h assays with no inhibitor. So, over 108 h of serial transfer without acetylene, there was a drop in *2H* utilisation by homoacetogenesis as compared to fresh rumen contents. *2H* utilisation by

propionate formation increased, as the propionate-producers might be able to grow well in the *in vitro* system compared to homoacetogens and methanogens. This may be due to production of lactate by *Streptococcus* spp., and subsequent fermentation of lactate to acetate and propionate by as-yet unclassified members of the order *Bacteroidales* or some other unknown bacteria.

When the acetylene-treated inoculum was used in the short-term assay (Figure 5.14c), *2H* utilisation by methanogenesis, hydrogen, butyrate, valerate and homobutyrogenesis all decreased as compared to fresh rumen contents (pre-treatment; Figure 5.13a). *2H* utilisation by homoacetogenesis was 2.32% in assays with no inhibitor and dropped to 1.69% with addition of BES. Importantly, however, *2H* utilisation by homoacetogenesis was greater when methanogenesis had been inhibited in the serial batch transfer system, showing that there was some potential for homoacetogenesis to increase when methanogens are inhibited. Homobutyrogenesis was very small and undetectable. *2H* utilisation by propionate was highest, at nearly 88.9% in all the three assays, i.e., with no inhibitor, and in the presence of BES or chloroform. This increase in *2H* utilisation by propionate formation in the short-term assay mirrors the results seen in the serial transfer experiment from which the inoculum was derived, an increase in amount and proportion of propionate formed (Figure 5.3). The relative abundance of *Streptococcus* spp. was very high at 108 h in the presence of acetylene (Figure 5.7b). Members of this genus are lactate producers. However, there was no increase in lactate concentration during the serial transfers in the presence of acetylene (Table 5.2), which implies that lactate might be used by other microbes that produce propionate, such as *Selenomonas* spp. (McInerney 1986) or as-yet unclassified members of the order *Bacteroidales*.

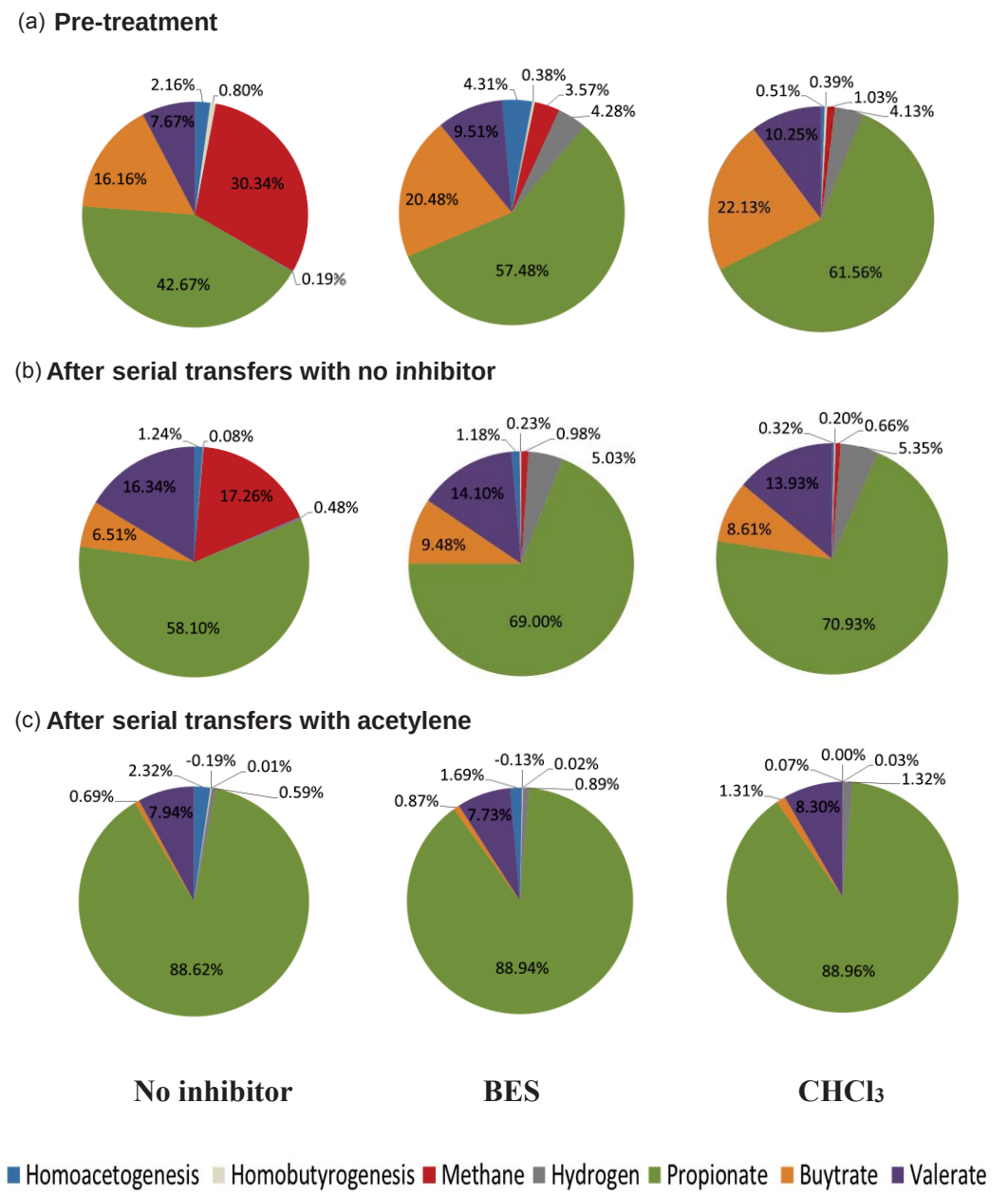

Figure 5.14 Electrons utilised (%) in production of various products *in vitro* over 8 hours by fresh sheep rumen contents (a) and after nine serial transfers without an inhibitor (b) or after nine serial transfers in the presence of acetylene (c). Each inoculum was tested in the short-term assay in the absence of inhibitor and in the presence of BES or chloroform. Data are presented as means ($n = 2$).

5.4.4.5.2 Electron recovery

The electron recovery was calculated as the ratio between *2H* utilised (equation 6) and *2H* produced (equation 5), as a percentage (equation 7):

2H % recovery = *2H* utilised × 100/*2H* produced (7)

The results are summarised in Table 5.2. Using fresh rumen contents, the electron recovery was on average 62.1%, irrespective of whether BES, chloroform or no inhibitor was added to the 8-h short-term assay. After nine serial batch transfers without acetylene, the electron recovery increased to approximately 80% in the assays with no inhibitor, or in the presence of BES or chloroform. Using the acetylene-treated inoculum, the electron recovery increased further to more than 95% in the assays with no inhibitor, BES or chloroform treatment. This increase in electron recovery may be due to the significant increase in *2H* utilisation by propionate over inhibition of methane production as depicted in Figure 5.13c. Presumably, other electron sinks that were not measured or could not be accounted for as accurately became less significant. Decreases in *2H* recovery with 100% inhibition of methane production as compared to control treatments have been reported for batch (95.2% to 57.6%) and continuous (67.9% to 46.1%) culture experiments using rumen fluid (Ungerfeld 2015). However, this does not appear to be the case in the present study, as electron recovery increased from approximately 62% in fresh rumen contents and approximately 80% in the serial transfers with no inhibitor to more than 95% after inhibition of methane production in the presence of acetylene in the serial transfers.

Table 5.2 Electron recovery in short-term incubations of fresh sheep rumen contents (pre-treatment), and of contents serially-transferred without and with acetylene.

	Mean electron recovery (%, ± SEM[a]; *n* = 2[b])		
Inhibitors added in short-term (8 h) assay	Pre-treatment (fresh rumen contents)	After serial transfers with no inhibitor	After serial transfers with acetylene
None	63.8 ± 0.34	81.1 ± 1.25	95.7 ± 0.24
BES	62.5 ± 0.12	79.9 ± 1.51	97.9 ± 1.90
$CHCl_3$	59.9 ± 0.10	79.9 ± 0.22	103.9 ± 6.53

[a]Standard error of the mean.

[b]No. of experimental replicates.

5.4.5 Discussion

In previous studies, increases in acetate formation were observed when homoacetogens or feed additives were added to *in vitro* systems (Chaucheyras *et*

al. 1995, Nollet *et al.* 1997, Le Van *et al.* 1998, Nollet *et al.* 1998, Lopez *et al.* 1999, Yang *et al.* 2015). However, the contribution of acetate formation from homoacetogenesis was not very clear. In the present study, the potential of resident homoacetogens to increase the contribution of homoacetogenesis to electron use in the absence of methanogenesis was studied in a serial batch system using acetylene as a methanogen-specific inhibitor. The end products, and their contribution to electron utilisation, were determined in the short-term assay developed in Chapter 4 using labelled CO_2 to study acetate derived from homoacetogenesis.

A decrease in methane production has been observed during *in vitro* incubation of sheep rumen contents using dissolved acetylene at concentration of 1.6 mM (Elleway *et al.* 1971). In the present study, acetylene at a concentration of 0.5 mM inhibited methane production completely over 108 h of *in vitro* serial batch fermentation experiment. Hydrogen was initially elevated but then decreased to be about 2.5-fold greater than in parallel experiments without acetylene after 108 h of serial transfer. This is comparable to another consecutive batch culture experiment using cow rumen fluid in the presence of 100 μM of acetylene. In that experiment, acetylene completely inhibited methane production over five days, although hydrogen was high for days 1 and 2, and then started decreasing over time (M. Tavendale, personal communication). Total gas decreased with acetylene addition in the present study, which is also comparable with the study done by Tavendale *et al*. (M. Tavendale, personal communication). Oremland and Taylor (1975) also reported inhibition of methane production and transient accumulation of hydrogen in the presence of 8.3 mM acetylene in marine sediments (Oremland & Taylor 1975). Increases in hydrogen accumulation have been observed during inhibition of methanogenesis in rumen fluid incubations using bromochloromethane and BES (Nollet *et al.* 1997, Goel *et al.* 2009). Acetylene has been reported to inhibit methane formation from anaerobic paddy soils (Raimbault 1975), waste biodegradation (Zhao *et al.* 2009) and lake sediments (Macgregor & Keeney 1973). In pure culture studies, acetylene has been observed to be effective inhibitor of methanogens even at a very low concentration range, without having any effects on other bacteria as demonstrated in Chapter 3 and by Sprott *et al.* (1982). In agreement with the more than 100-fold decrease in methane formation as compared to serial transfers with no inhibitor, total archaeal numbers also decreased by more than 100-fold over 108 h of acetylene treatment. This also resulted in changes in archaeal

community structure, leading to a decrease in the relative abundance of members of the *Methanobrevibacter gottschalkii* clade, and increases in *Methanosphaera* sp. Group 5 and *Methanosphaera* sp. ISO3-F5.

Acetylene addition also resulted in an increase in propionate, and decrease in total acetate and butyrate formation over time. An increase in propionate and a decrease in acetate has also been observed in number of other studies involving inhibition of methane production and is attributed to a shift in the use of reducing equivalents (Van Nevel *et al.* 1974, Nevel & Demeyer 2007). This has been widely discussed (Janssen 2010, Ungerfeld 2013, 2015). Decreases in butyrate formation have been observed during incubation of rumen contents for 48 h in the presence of antimethanogenic compounds such as propynoic acid, 2-nitroethanol and sodium nitrate (Zhou *et al.* 2011). The amount of valerate formed decreased in the presence of acetylene as compared to the no inhibitor serial transfers. The amounts of isobutyrate, isovalerate and caproate formed were relatively very small, and sometimes even undetectable. Only formate and ethanol were detected during acetylene treatment, but the amounts present were also very small. The absolute bacterial numbers were stable even after inhibition of methane production, but the bacterial community composition was changed, which must have led to the changes in VFA formation and decrease in hydrogen production.

Prevotella spp. represented the most dominant genus, as also observed in previous studies (Bekele *et al*. 2010, Pitta *et al*. 2010; Zened *et al*. 2013). The relative abundance of *Prevotella* spp. first increased, and then decreased over time with inhibition of methane production using acetylene. The relative abundance of *Prevotella* spp. has also been observed to decrease with inhibition of methane formation during addition of cashnew nut shell extract in *in vitro* experiments using rumen fluid (Danielsson *et al.* 2014, Shinkai *et al*. 2012) and also during inhibition of methane production in goats using bromochloromethane (BCM; Mitsumori *et al*. 2012). The relative abundance of unclassified members of the order *Clostridiales*, unclassified members of the families *Ruminococcaceae* and *Veillonellaceae* decreased, while *Pseudobutyrivibrio* spp. and *Fibrobacter* spp. increased significantly over time in the presence of acetylene at 108 h compared to non-inhibited incubations. These are the typical fermentative bacteria which are thought to be responsible for the majority of feed fermention in the rumen (Henderson *et al.*

2015). The relative abundance of *Streptococcus* spp. increased significantly over time, to become the most abundant taxon. However, there was no lactate accumulation during serial transfers in the presence of acetylene. The lactate was presumably utilised by other bacteria that ferment lactate to propionate, such as *Selenomonas* spp., *Megasphaera elsdenii* and *Veillonella alcalescens* (McInerney 1986) or members of the order *Bacteroidales* (Macy *et al.* 1978, Schultz & Breznak 1979). *Selenomonas* spp. were observed with inhibition of methane production, however it did not increase significantly and eventually decreased over time. At present, the bacteria that ferment lactate to propionate remain unknown.

Changes were also observed in gas production, VFA profile and microbial community composition during serial transfers without inhibitor. The amount of methane formed halved during serial transfer, which is in agreement with the two-fold decrease in absolute archaeal numbers. There was also a change in archaeal community structure. Members of the *Methanobrevibacter gottschalkii* clade formed the major archaea at the start, as expected since they are usually the dominant archaea present (Janssen & Kirs 2008). However, over time their relative abundance reduced, and there was increase in the relative abundance of *Methanomassiliicoccales* Group 9 sp. ISO4-G1 and *Methanomassiliicoccales* Group 12 sp. ISO4-H5. The amount of hydrogen gas formed increased by four-fold and total gas decreased by 14% after 108 h of serial transfers. The VFA profile also shifted towards decreased acetate and increased propionate, even though no inhibitor was used. This can also be linked to changes in bacterial community structure during the serial transfers without inhibitor, although the total bacterial numbers remained stable. There was a first increase, and then a decrease in the relative abundance of *Prevotella* spp., and increases in the relative abundance of *Streptococcus* spp., unclassified members of the order *Bacteroidales* and unclassified members of the family *Lachnospiraceae*. The relative abundance of unclassified members of order *Clostridiales* and families *Ruminococcaceae* and *Veillonellaceae* decreased, whereas the relative abundance of *Pseudobutyrivibrio* spp. increased in the non-inhibited incubation over time. The increase in the relative abundance of *Streptococcus* spp. did not led to increase in lactate accumulation in the non-inhibited serial transfers. All of these changes, even in the absence of methane inhibitor, can be attributed to serial transfers after every 12 h, as only 33% of the diluted rumen contents were transferred. In this way, the serial transfer

system did not completely mimic a rumen system, although all the organisms present were known to be rumen-dwelling microbes. The effects of methanogen inhibition were also similar to those expected from studies with animals. There was a large reduction in methane formation, and increase in net hydrogen production, and a change in VFA towards more propionate and less acetate. The system could be used to investigate the response of homoacetogenesis to inhibition of methane production. This was done by using the 8-h assay developed in Chapter 4 to measure labelled acetate formation from $^{13}CO_2$.

Homoacetogenesis increased in fresh rumen contents prior to serial transfer when methane formation was inhibited using BES for 8 h. This can be attributed to an immediate response to the increase in hydrogen concentration after methane inhibition and is comparable to the findings reported in Chapter 4. After 108 h of serial transfers in the absence of inhibitor, homoacetogenesis was lower in the *in vitro* short-term assay that it was using fresh rumen contents. This suggests that homoacetogens were disadvantaged in the serial transfer system. The calculated dissolved hydrogen concentrations were very low during the serial transfers without inhibitor (2.94 μM), probably too low to allow the homoacetogens to expand.

After 108 h of serial transfers in the presence of acetylene, homoacetogenesis was comparable to that measured in fresh rumen contents in assays with no inhibitor. Importantly, however, it was nearly twice that measured in the transfers without acetylene. The dissolved hydrogen concentrations in the serial batch system with acetylene were 42.0 μM at 12 h and 30.4 μM at 108 h, which is higher than without the inhibitor, and the expected relative rate of metabolism of homoacetogens was therefore also higher. The potential increase in homoacetogenesis was also consistent with the electron use by homoacetogenesis, which was 2.16% of total electron utilisation in fresh rumen contents, which was 1.24% of total electron utilisation after 108 h in the absence of acetylene and 2.32% after serial transfers in the presence of acetylene. This showed that there was some potential for homoacetogenesis to increase when methanogens are inhibited.

The contribution of propionate to *2H* utilisation increased from approximately 42% in fresh rumen contents to approximately 88% after 108 h of inhibition of methane production with acetylene, and is in accordance with the

increase in the amounts and proportions of propionate formed over inhibition of methane production in the serial transfer experiment. The electron utilisation by propionate increased by 1.53-fold in the presence of acetylene over serial transfers, in good agreement with the 1.43-fold increase after nine serial transfers. During another *in vitro* rumen fermentation study with two contrasting diets and inhibition of methanogenesis, there was also a dramatic increase in the electron flow to propionate from 26.7% to 61.7% (O'Brien *et al.* 2014, Ungerfeld 2015). In batch and continuous culture experiments, inhibition of methane production by ionophores (monensin), linoleic and linolenic acids, and cashew nut shell liquid resulted in increases in *2H* utilisation by propionate (Chalupa *et al.* 1980, van Nevel & Demeyer 1981, Watanabe *et al.* 2010, O'Brien *et al.* 2014, Ungerfeld 2015). This suggests that propionate is one of the major electron sinks when methanogenesis is inhibited, as we also observed increase in propionate production over inhibition of methane production.

5.5 Summary and perspectives

The present study was designed to determine the effect of inhibiting methane production using acetylene as an inhibitor of methanogens during *in vitro* incubation of sheep rumen fluid, particularly the effect on homoacetogenesis. The addition of acetylene over 108 h during *in vitro* serial batch fermentation inhibited methane production. With the addition of acetylene, the VFA profile shifted towards more propionate and less acetate. The microbial community was also affected by the presence of acetylene. There was a dramatic decrease of more than 100-fold in total archaeal numbers over inhibition of methane production and two-fold decrease in no inhibitor incubations in the serial batch system, whereas the total bacterial numbers remained stable. The relative abundance of different members of both the archaeal and bacterial communities changed over time in the presence and absence of acetylene. The archaeal and bacterial community differed in both no inhibitor and acetylene treatment, and changed over serial transfers. The animal provides a continuous process of removing VFA and other end-products of fermentation, but this happened only every 12 h in the *in vitro* system. For this reason, the serial batch system probably does not represent changes in response to a methane inhibitor in the rumen very well. The serial batch system did, however, provide a means of studying the impact of inhibition of methane production on

homoacetogenesis, although care must be taken knowing that the system did not represent a real rumen.

The results of this investigation confirmed the occurrence of homoacetogenesis in fresh sheep rumen fluid as also observed in Chapter 4. When methanogenesis was inhibited over 108 h during *in vitro* serial batch fermentation using acetylene, homoacetogenesis increased as compared to no inhibitor serial transfers. The hydrogen concentration increased after 108 h of serial transfers in the presence of acetylene, and the calculated relative rate of metabolism for homoacetogens also increased as compared to no inhibitor transfers, accounting for increase in homoacetogenesis. This further lead to increase in *2H* utilisation by homoacetogenesis from 1.24% in no inhibitor serial transfers to 2.32% in the presence of acetylene. However, in the absence of methanogenesis during serial transfers, the major electron sink was observed to be propionate, with a 1.53-fold increase in *2H* utilisation by propionate after inhibition of methane production over serial transfers.

These results suggest that inhibiting methane production in sheep rumen fluid incubated *in vitro* during serial transfers increased homoacetogenesis. The bacteria responsible were clearly able to maintain themselves in this artificial environment. Acetylene will now be used to inhibit methanogenesis in sheep (*in vivo*), and detect if resident homoacetogenesis could increase, when provided a more favourable environment (the rumen), and determine if they can take over *2H* utilisation in the absence of methanogenesis.

Chapter 6

Effect of inhibition of methanogenesis on fermentation in sheep rumen and homoacetogenesis

6.1 Introduction

Homoacetogens can use H_2 to reduce CO_2 to acetate, and may compete with other microbes using hydrogen and so act as an alternative hydrogen sink. It would be beneficial for ruminants if homoacetogenesis (i.e., reductive acetogenesis) was a means of hydrogen utilisation in the absence of methanogens, as the acetate produced can be used as an energy source for the animal, instead of feed energy being lost in the form of methane. Few studies have been conducted on the significance of homoacetogenesis in ruminants (see Chapter 1; Section 1.4.5). The presence of homoacetogens in the rumen of 24-h-old lambs was shown in culturing and labelling experiments, where labelled CO_2 was incorporated into acetate, while no labelled methane was formed (Morvan *et al.* 1994). In the developing rumen of lambs, where methanogens had yet to be established, homoacetogens reached densities of 10^7 to 10^8 cells per g of rumen contents and appeared to use hydrogen to produce acetate (Fonty *et al.* 2007). In another study on 17-h-old lambs raised aseptically, establishment of methanogens did not alter the diversity of homoacetogens (Gagen *et al.* 2012). An attempt was made to use BES to induce reductive acetogenesis in the rumen of a fistulated sheep, but failed, presumably due to the adaptation of methanogens to BES (Immig *et al.* 1996). As discussed in Chapter 5, incubation of sheep rumen contents with the methanogen inhibitor acetylene *in vitro* over 108 h did not result in a significant increase in homoacetogenesis even in the absence of methanogens, however it did increase as a percentage of total acetate. This may have been due to the serial dilution culture technique, where the rumen contents were diluted to one-third with fresh buffer and substrate after every 12 h. This may not provide conditions suitable for an increase in homoacetogenesis. The next step was to study if homoacetogenesis can act as an alternative electron sink to methanogenesis in animals, when provided a favourable environment (the rumen) and the opportunity to take over hydrogen utilisation in the absence of methanogenesis.

Based on the studies referred to above, we know that reductive acetogenesis can be induced in ruminants if methanogens could be inhibited using an inhibitor other than BES, which failed due to adaptation of methanogens to BES over 4 days (Immig *et al.* 1996). Chloroform and bromochloromethane have been used in the past as methane inhibitors. Chloroform is known to inhibit methanogenesis in sheep and cow rumens, both *in vitro* and *in vivo* (Lanigan 1972, Knight *et al.* 2011), and inhibits homoacetogens (see Chapter 3). Bromochloromethane has been shown to inhibit methane formation in ruminants (May *et al.* 1995, McCrabb *et al.* 1997, Mitsumori *et al.* 2012), but its use is not permitted in New Zealand. It is a halogenated methane analogue, like chloroform, and might also be expected to inhibit homoacetogens.

Research carried out at AgResearch has explored the utility of acetylene as a methane inhibitor for experimental purposes, and demonstrated that 100% inhibition of methane production could be achieved when sheep were administered an acetylene-generating bolus (M. Tavendale, personal communication). Therefore, in the present study, the aim was to inhibit methanogens in sheep using acetylene as a methanogen-specific inhibitor, and determine if there is an increase in homoacetogenesis when methanogens are inhibited. This would allow an estimation of the contribution of homoacetogens to hydrogen (electron) utilisation in the absence of methanogens in sheep. Other changes in the rumen, such as changes in volatile fatty acid (VFA) concentrations and microbial community composition were examined. The diversity of formyltetrahydrofolate synthetase (FTHFS) genes (*fhs*), that code for the enzyme that catalyses the reductive step in the Wood-Ljungdahl pathway, was assessed as a marker for homoacetogens, by comparison to a FTHFS profile hidden Markov model (Henderson *et al.* 2010). Chloroform was administered to another group of sheep as an additional treatment to study what happens to hydrogen when both methanogens and homoacetogens are inhibited.

6.2 Objectives

The aims of the present study were:

- To inhibit methane production using acetylene and chloroform administered into sheep rumens, and measure methane emissions using respiratory chambers

- To measure the shifts in VFA concentrations in the rumen during inhibition of methane production with acetylene and chloroform
- To determine the changes and shift in archaeal and bacterial communities in the rumen when methane formation is inhibited
- To identify FTHFS sequences in rumen samples using a homoacetogen FTHFS profile hidden Markov model (HoF-HMM)
- To measure homoacetogenesis in sheep rumen fluid incubated *in vitro* prior to and during inhibition of methanogenesis in sheep rumen fluid by incorporation of $^{13}CO_2$ into ^{13}C-acetate as described in Chapter 4, i.e., in a short-term *in vitro* homoacetogenesis assay
- To measure the production of other fermentation products such as total gas, methane and hydrogen, and VFA by sheep rumen fluid, using the short-term assays
- To study the contribution of homoacetogenesis and other alternative hydrogen utilisers towards the flow of electrons in sheep rumen fluid prior to and during inhibition of methane, using the short-term *in vitro* assays

Summarizing the above objectives, we aimed to establish, in response to five days of sustained inhibition of methanogenesis, whether the homoacetogenic community resident in the rumen takes over the role of rumen hydrogen use previously occupied by the methanogens prior to their inhibition.

6.3 Summary of materials and methods

An overview of the trial, with variables measured, is given in Figure 6.1.

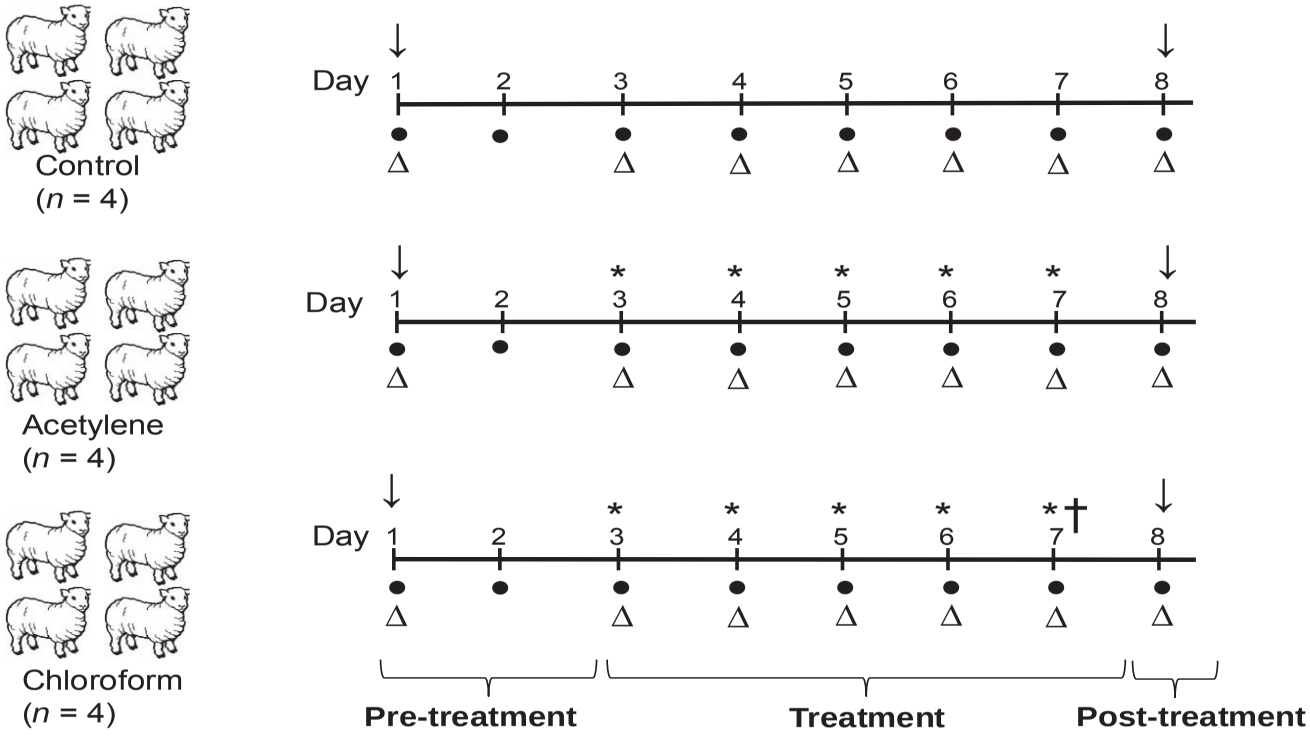

Figure 6.1 Overview of the animal trial.

* Sheep received acetylene or chloroform.

● Methane measured in respiratory chambers.

Δ Rumen samples collected from sheep to determine fermentation variables (VFA) and microbial community composition (archaeal and bacterial 16S rRNA gene numbers, relative abundance of taxa, and FTHFS gene composition).

↓ Short-term *in vitro* homoacetogenesis assay to measure homoacetogenesis as described in Chapter 4.

† One sheep was removed from the trial due to health issues.

6.3.1 Animals and diet

Based on rumen *in vitro* fermentations carried out earlier, on three separate occasions, the fractional rate of homoacetogenesis was determined and these data were used in a power analysis to determine the probability of obtaining significant results. Based on the power analysis, twelve fistulated sheep (wethers; 2-3 years old; weight in the range of 60-95 kg) were used. They were housed in pens and adapted to a general purpose (GP) diet for two weeks. A detailed description of further materials and methods is provided in Chapter 2. After diet adaptation for two weeks, the sheep were equally divided into three groups: control (n = 4), acetylene (n = 4) and chloroform (n = 4) groups. The control group were used to provide an experimental covariant. The sheep were then transferred into metabolism crates and housed continuously in the methane respiratory chambers to measure their daily methane gas emissions for 7 days (Figure 6.1; Pinares-Patiño *et al.* 2008). These chambers measure carbon dioxide, methane, and hydrogen concentrations in the exit gas from each chamber at approximately 6.4 min intervals, by sampling gas sequentially from each chamber. Coupled with a measured gas flow, this allows calculation of gas emissions. However, acetylene in the exhaled breath of the acetylene-treated sheep was detected by the hydrogen detector, and so hydrogen data were not collected. Table A.3.1 in the Appendix 3 provides details of animal weights, dry matter intake (DMI), and feed refusals, and the gas production from each sheep. All aspects of the trial were approved by the Grasslands Animal Ethics Committee, under approval AEC#12908. One of the chloroform-treated sheep had to be withdrawn from the experiment, and so the data from that group are from the remaining three sheep.

6.3.2 Administration of inhibitors and sampling from sheep

Control, acetylene and chloroform treatments were administered to the sheep from day 3 to day 7 in the morning around 9 a.m. via the fistula. A slow-release acetylene bolus was prepared using barium sulfate, iron powder, beeswax and calcium carbide (M. Tavendale, personal communication). Chloroform was complexed with 1.8 g of cyclodextrin paste and added to a capsule, and dosed directly into the rumen fistula. Control sheep received a non-acetylene generating bolus containing barium sulfate, iron powder, beeswax and calcium carbonate. Control sheep also received 1.8 g of cyclodextrin enclosed in a capsule. Detailed descriptions of the preparation

of the control, acetylene and chloroform treatments are provided in Chapter 2. Rumen samples (50 ml) were collected through the fistula daily in the morning, before administration of the inhibitors, for VFA and microbial community analyses (Figure 6.1). Nearly 200 ml of rumen sample was collected in the morning of day 1 (pre-treatment) and day 8 (while methane production was inhibited), for short-term *in vitro* homoacetogenesis assays to determine homoacetogenic activity in the rumen, and the ability of the rumen contents to produce the different VFA, methane, hydrogen and total gas, as described in Chapter 4, and using additional controls (3 mM BES and 100 μM chloroform; Figure 6.1).

6.3.3 Analysis of rumen samples

The rumen samples collected from sheep were analysed for the following:

- VFA by gas chromatography with a flame ionisation detector (GC-FID) and high-performance liquid chromatography (HPLC)
- microbial community structure (archaeal and bacterial 16S rRNA gene numbers by qPCRs, relative abundance of archaeal and bacterial taxa by 454 pyrosequencing after PCR amplification, and analysis of formyltetrahydrofolate synthetase (FTHFS) diversity by Sanger sequencing after PCR amplification and cloning in *Escherichia coli*
- homoacetogenesis on day 1 (pre-treatment) and on day 8 (while methane production was inhibited) in the *in vitro* short-term assay system via incorporation of $^{13}CO_2$ into ^{13}C-acetate (Chapter 4), with samples analysed using gas chromatography-isotope ratio mass spectrometry (GC-IRMS), as well as measurement of other fermentation variables such as methane, hydrogen, total gas (GC) and VFA (GC-FID)

6.3.4 Statistical analysis of data from animal trial

There were three treatments (control, acetylene and chloroform) with 4, 4 and 3 experimental units (animals) respectively, each repeatedly observed and measured over 8 time points (day 1 to day 8). There were several responses and each was analysed separately.

6.3.4.1 Repeated measures ANOVA

Since measurements were made on the same experimental unit (animal), a *repeated measures ANOVA* with one experimental factor (3 treatments; control, acetylene

and chloroform) and one repeated factor (8 time points; day) was considered. Animals were treated as genuine replications. Linear mixed effects models with Treatment, Day and Treatment:Day (interaction) as fixed effects and 'Animal' as random effect were utilized with a covariance structure for the random effect that accounts for the repeated nature of measurements made on each animal. AIC (Akaike information criterion) was utilised to choose the optimal covariance structure. P-values for paired comparisons were adjusted by the Bonferroni method. However, for total archaeal and bacterial numbers the use of Fisher Least Significant Difference (LSD) was justified due to the small number of comparisons. The significance of all effects were set at 5% ($p < 0.05$), and the effects with p-values more than 5% and less than 10% were regarded as a trend towards an increase or decrease ($p = 0.05$ to 0.10).

6.3.4.2 Transformations

When 'ANOVA assumptions' are not satisfactory when analysing the raw data (usually via examination of model residuals), the alternative is to base the inference on the 'transformed data' (and/or use resampling methods such as the "permutation tests" (also sometimes known as 'randomization tests')). Simple transformations such as SQRT (square root) and LOG (logarithm) were attempted. For those variables meeting the ANOVA assumptions only marginally, permutation tests (with 5000 randomizations) were evaluated and if the resulting significance of the various effects were very similar to those of the usual ANOVA, the results of the ANOVA were retained; otherwise, the permutation test results retained.

6.3.4.3 Software

All analyses were carried out using the R software version 3.3.0 and packages such as *nlme* (and its *lme* function) and *predictmeans* (Pinheiro & Bates 2000, R Core Team 2016).

6.3.4.4 Principal component analysis

Principal component analysis (PCA) was based on Eigen-analysis of a correlation matrix using Minitab software (version 17; Minitab Inc., Pennsylvania, USA). The inputs were the relative abundance data for each genus. Where taxa were not be defined below the family or order level, those groups were used in the analysis as if they were genera.

6.3.5 Statistical analysis of data from short-term *in vitro* homoacetogenesis assay

Data were compared using one way ANOVA to compare treatment followed by Dunnett's multiple comparison with control using the Minitab software analysis tool (version 17; Minitab Inc., Pennsylvania, USA).

6.4 Results and discussion

Based upon previous studies using acetylene and chloroform as methane inhibitors, it was expected that methane emissions from sheep would decrease with administration of acetylene or chloroform, and that there would be changes in rumen fermentation variables and microbial community structure. There was an expectation of increased acetate formation from homoacetogenesis in response to inhibition of methane production with acetylene, as hydrogen that was being used for methane formation might now be used by homoacetogenesis. Some expected effects of the inhibitors in sheep are described in Table 6.1.

Table 6.1 Expected effects of inhibitors in sheep rumen.

Inhibitors	Effects
None	Normal methanogenesis and homoacetogenesis
Acetylene (approximately 3 mM)	Inhibit methanogenesis and increase homoacetogenesis
Chloroform (approximately 300 μM)	Inhibit methanogenesis and inhibit or decrease homoacetogenesis

6.4.1 Acetylene and chloroform inhibited methane production in sheep

Methane production by sheep was followed over time in respiratory chambers. Methane yields (g CH_4 per kg of DMI) were decreased significantly in sheep that had received acetylene (98.7%; p = 0.0001) or chloroform (91.0%; p = 0.0001; Figure 6.2b and 6.2c) from day 4 to day 8 as compared to day 2 (pre-treatment), whereas there was no significant affect in control sheep over time (p = 1.000; Figure 6.2a). A detailed description of each sheep weight, DMI, feed refusals, methane and carbon dioxide produced by each sheep per day is provided in the Appendix 3 (Table A.3.1). Acetylene treatment seemed to have some effect on sheep, so that

the amounts of feed refusals by each sheep decreased by approximately 51% on average after five days of treatment. Therefore, measurements of homoacetogenesis were made at that time and the experiment could not be extended further.The mean amounts of methane and DMI of control, acetylene- and chloroform-treated sheep over time have also been provided in Appendix 3 (Table A.3.3).

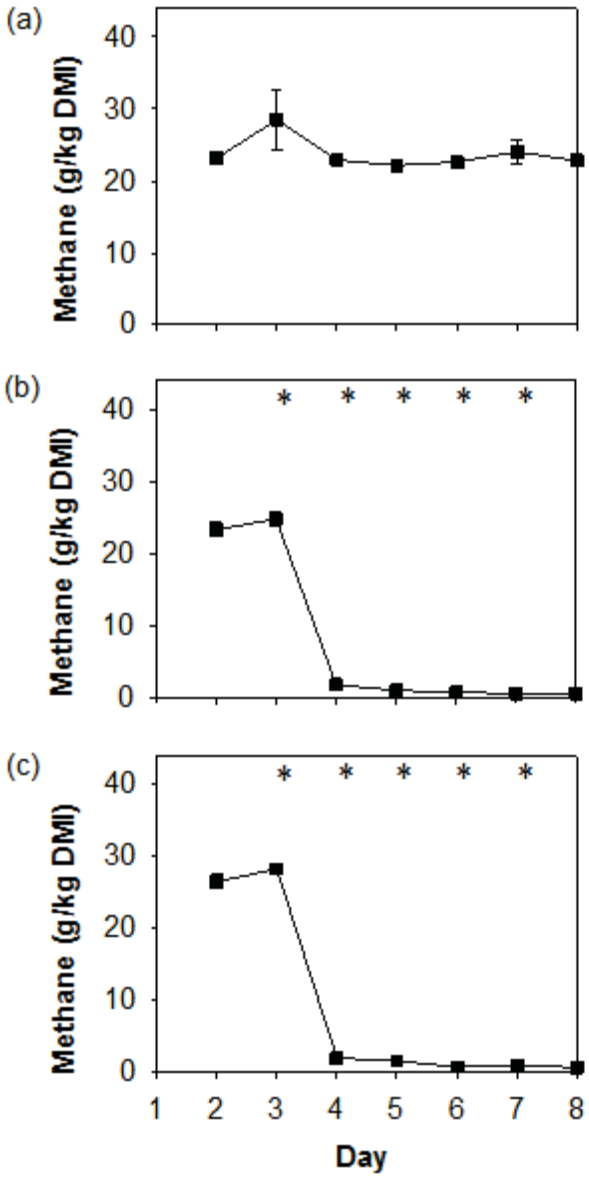

Figure 6.2 Daily methane yields from sheep that received no inhibitor (a) and sheep that were dosed with acetylene (b) or chloroform (c). The methane yields are calculated from the amount of methane emitted in the preceding 24 h (approximately 9 a.m. to 9 a.m.), and the amount of feed consumed in the same period. The * marks the days sheep received inhibitors. Data are presented as means ± standard errors of the mean (control and acetylene sheep, $n = 4$; chloroform sheep, $n = 3$).

6.4.2 Volatile fatty acid profile of sheep changed following inhibition of methanogenesis

VFA concentrations in sheep rumen samples were determined to see if there was a change in fermentation patterns following the administration of acetylene (Figure 6.3b) or chloroform (Figure 6.3c) compared to the controls (Figure 6.3a). The concentration of acetate was not significantly changed in the rumen of acetylene-treated sheep from day 4 to day 7 ($p = 1.000$), however it decreased by 37.6% at day 8 ($p = 0.016$) relative to day 1 (pre-treatment). This may have been partly caused by the increasing amount of feed refusals by these sheep as the experiment progressed. In the chloroform-treated sheep, acetate was not significantly different even at day 8 ($p < 1.000$), compared to day 1 (pre-treatment). Propionate concentrations increased significantly ($p < 0.007$) by more than 67.9% in acetylene-treated sheep immediately after treatment commenced, but at day 8 the concentration of propionate was not significantly different ($p = 1.000$) from that at day 1 (pre-treatment). In chloroform-treated sheep, the concentration of propionate also increased significantly over time from day 4 to day 7 ($p < 0.040$) by more than 92.1%, but at day 8 ($p = 0.133$) it was not significantly different from day 1. The concentration of butyrate was not significantly affected even at day 8 of treatment in both acetylene-treated sheep ($p = 1.000$) and chloroform-treated sheep ($p = 1.000$) as compared to day 1 (pre-treatment). Overall, the total ruminal VFA concentrations were unaffected in both acetylene- ($p = 1.000$) and chloroform-treated ($p = 1.000$) sheep as compared to day 1 (pre-treatment) of the same treatment group. The lack of statistical significance was probably a result of the animal-to-animal variation, and the small number of animals used, especially in the chloroform treatment.

The proportions of the some VFAs seemed to be strongly affected by the treatments. In control sheep, acetate made up a large proportion of total VFA during the trial with no significant difference over time ($p = 1.000$; Figure 6.3d). In acetylene-treated sheep, acetate became proportionally less prevalent ($p = 0.0002$) and propionate became proportionally more prevalent ($p = 0.0001$) among the total VFA formed over time at day 8 as compared to day 1 (pre-treatment), while the proportion of butyrate was not significantly affected ($p = 1.000$, Figure 6.3e). In chloroform-treated sheep as well, acetate became proportionally less prevalent ($p =$

0.0001) and propionate became proportionally more prevalent (p = 0.0001) among the total VFA formed over time at day 8 as compared to day 1 (pre-treatment), while the proportion of butyrate was not significantly affected (p = 1.000; Figure 6.3f).

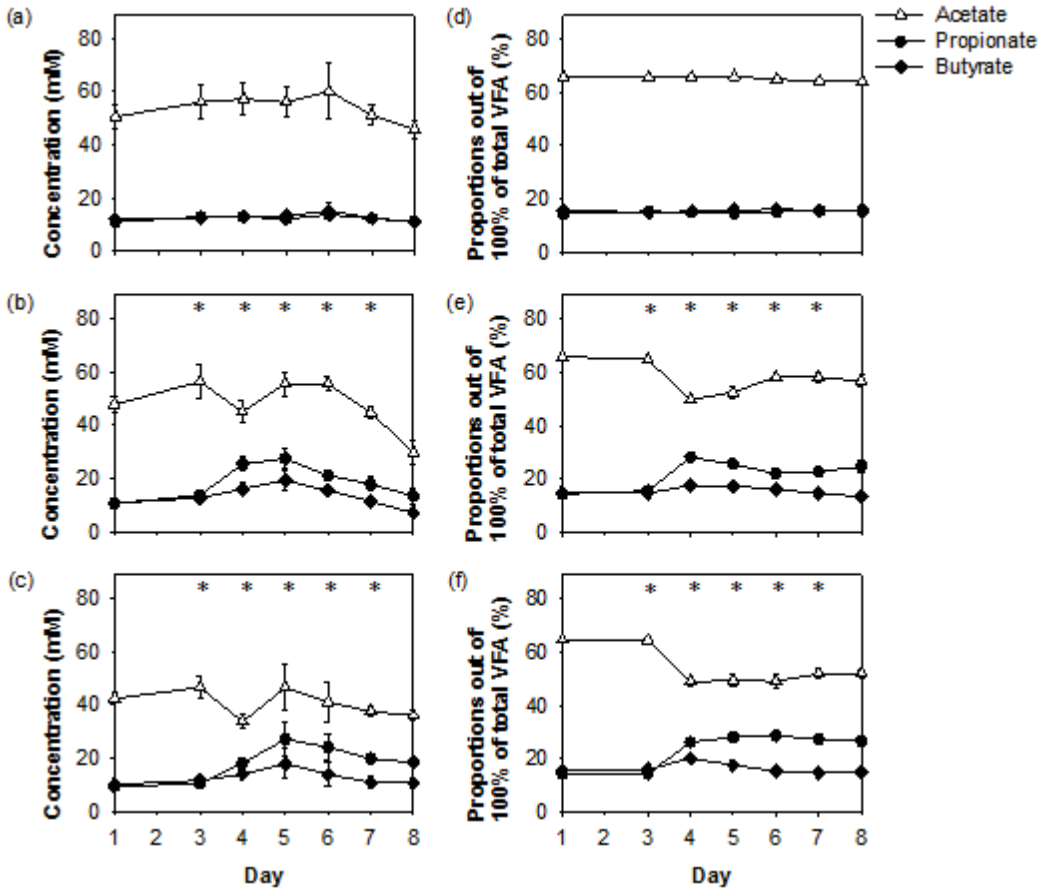

Figure 6.3 Volatile fatty acid concentrations (a; b; c) and proportions (d; e; f) of each VFA (acetate, propionate and butyrate) in sheep rumen contents that received no inhibitor (a; d) and that were dosed with acetylene (b; e) or chloroform (c; f). The values are the concentrations or proportions in rumen samples collected at approximately 9 a.m. on the day noted. The * marks the days sheep received inhibitors. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, *n* = 4; chloroform-treated sheep, *n* = 3).

Branched and short-chain fatty acids were also measured. Isobutyrate (p = 1.000), isovalerate (p = 1.000), caproate (p = 1.000) and valerate (p = 1.000) were not significantly different in control sheep at day 8 as compared to day 1 (Figure 6.4a). Similarly, in acetylene-treated sheep as well, isobutyrate (p = 0.895), isovalerate (p = 0.904), caproate (p = 0.255) and valerate (p = 1.000) seemed unaffected at day 8 as compared to day 1 (Figure 6.4b). In chloroform-treated sheep, there were no shifts in branched and short-chain fatty acid concentrations over time

and at day 8 (isobutyrate ($p = 1.000$), isovalerate ($p = 1.000$), valerate ($p = 1.000$), and caproate ($p = 1.000$)), as compared to day 1 (pre-treatment; Figure 6.4c).

Lactate, formate and ethanol concentrations in the rumen samples were also measured. However, these were detected at very small concentrations (Appendix 3; Table A.3.2). Small amounts of lactate were detected in all animals, but mostly less than 0.01 mM and these did not differ significantly in acetylene- and chloroform-treated sheep compared to day 1 (pre-treatment) and showed same response ($p = 1.000$). Formate increased in chloroform-treated sheep from day 4 to day 8 by approximately 10 to 18-fold and was significantly different ($p = 0.0001$) from day 1. However, there was no significant difference in acetylene-treated sheep ($p = 0.653$) at day 8 compared to day 1 (pre-treatment). There was also no significant difference in ethanol in either acetylene- ($p = 1.000$) or chloroform-treated ($p = 1.000$) sheep as compared to day 1 (pre-treatment) in the same group of sheep. Overall, the amounts of these three products formed were very small, and these were not considered further for any calculations.

The mean concentrations and proportions of each VFA in control, acetylene- and chloroform-treated sheep over time are provided in Appendix 3 (Table A.3.2).

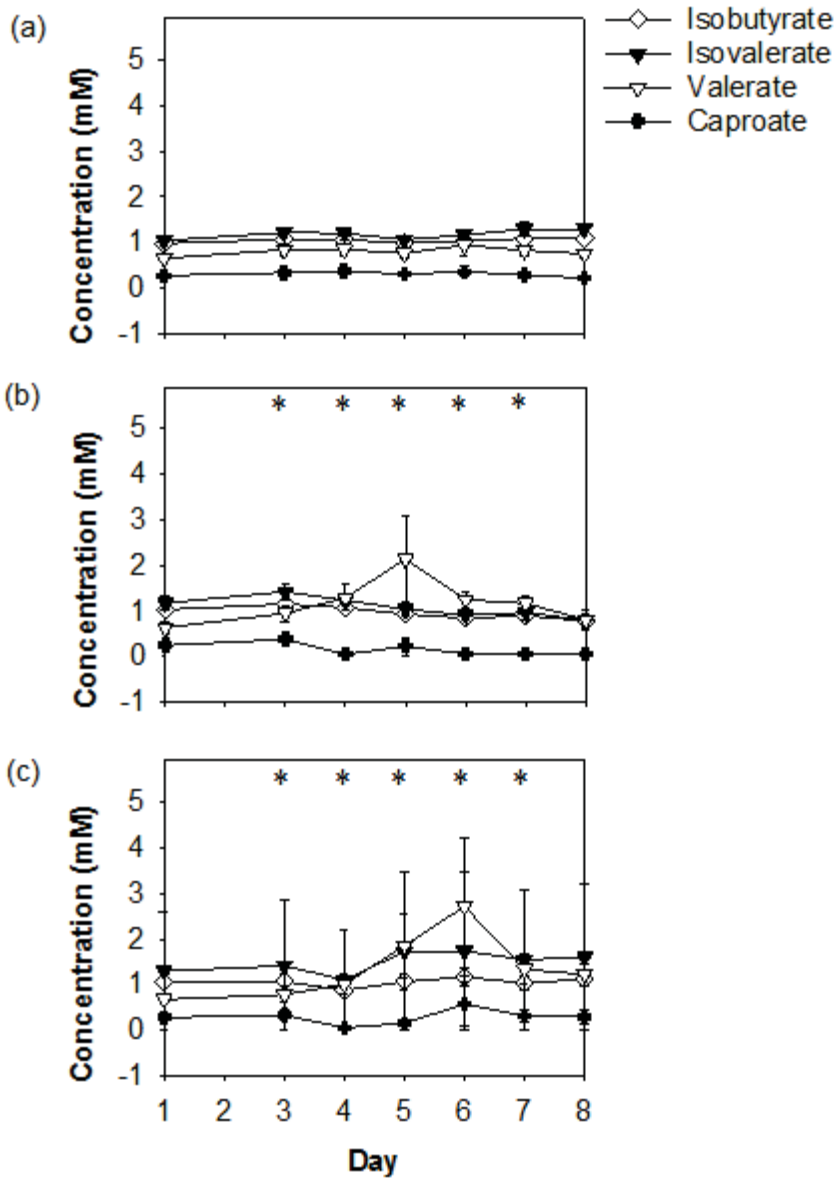

Figure 6.4 Branched and short-chain fatty acids (isobutyrate, isovalerate, valerate and caproate) concentrations in rumen contents of sheep that received no inhibitor (a) and sheep that were dosed with acetylene (b) or chloroform (c). The values are the concentrations in rumen samples collected at approximately 9 a.m. on the day noted. The * marks the days sheep received inhibitors. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).

6.4.3 Archaeal and bacterial communities changed following inhibition of methane production

Total archaeal numbers decreased more than 10-fold following administration of acetylene ($p = 0.0001$) and chloroform ($p < 0.013$) at days 4 to 8 as compared to day 1 (Figure 6.5b and 6.5c). There was no difference over time in control sheep that received no inhibitor ($p = 1.000$; Figure 6.5a). Total bacterial numbers, in

contrast, remained stable and significantly unaffected at days 4 and 8 in acetylene-treated (p = 0.085 to 0.909) and chloroform-treated (p = 0.068 to 0.875) sheep as compared to day 1 (pre-treatment).

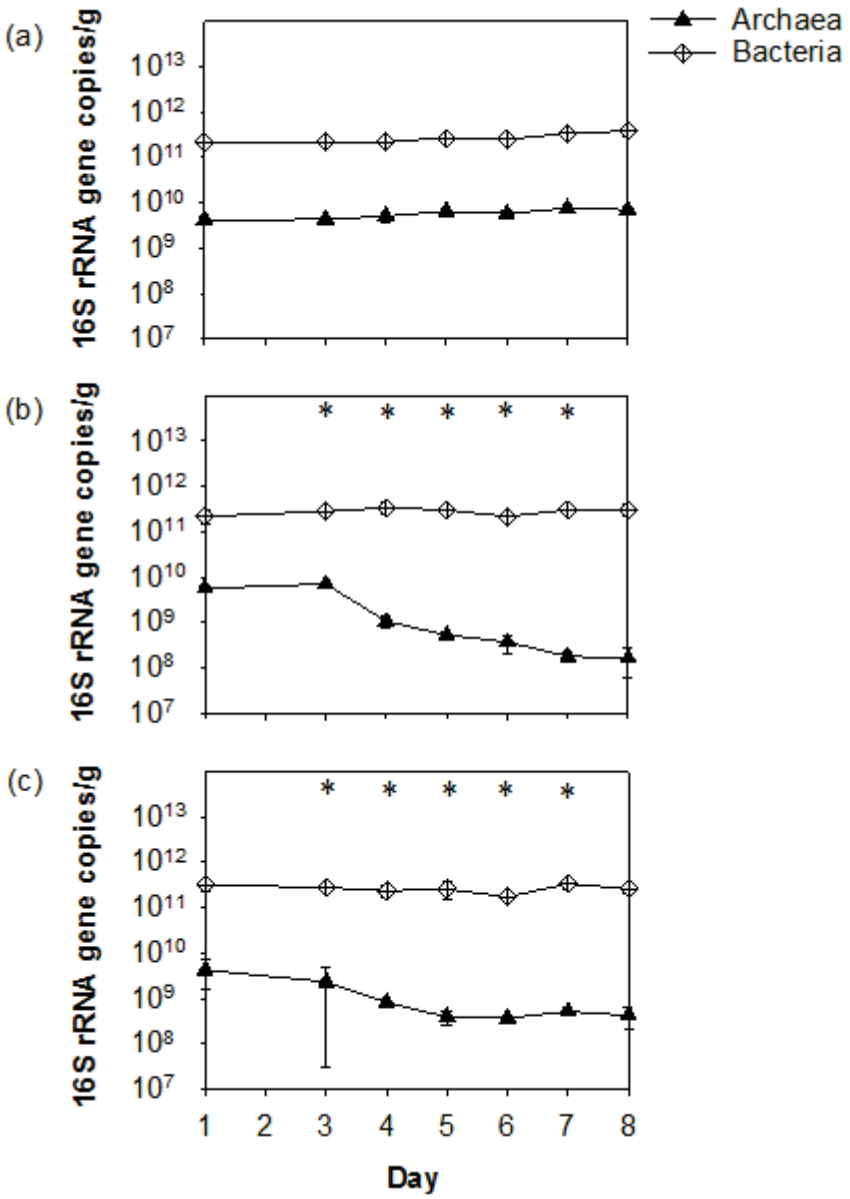

Figure 6.5 Absolute rumen archaeal and bacterial 16S rRNA gene numbers per g of freeze-dried rumen contents of sheep that received no inhibitor (a) and that were dosed with acetylene (b) or chloroform (c). The values are the concentrations in rumen samples collected at approximately 9 a.m. on the day noted. The * marks the days sheep received inhibitors. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, *n* = 4; chloroform-treated sheep, *n* = 3).

No significant changes were observed in relative abundance of the major taxa in archaeal communities over time in control sheep that received no inhibitor

(p = 1.000; Figure 6.6a), in agreement with the lack of change in methane production (Figure 6.2a) and total archaeal numbers (Figure 6.5a). Concomitant with the reduction in methane (Figure 6.2b and 6.2c) and absolute archaeal numbers in acetylene-treated and chloroform-treated sheep (Figure 6.5b and 6.5c), the relative abundance of different taxa in the archaeal communities in the rumens of the sheep changed drastically (Figure 6.6b and 6.6c). There was a large decrease in the relative abundance of 16S rRNA genes assigned to the *Methanobrevibacter gottschalkii* clade in both acetylene- (75.9%; p = 0.0001) and chloroform-treated (95.7%; p = 0.0001) sheep at day 8 as compared to day 1 (pre-treatment). In contrast, the relative abundance of members of the *Methanobrevibacter ruminantium* clade decreased in acetylene-treated sheep from day 6 to 7 (81.5% to 90.1%; p < 0.020), but it was not significantly different at day 8 (p = 0.631) as compared to day 1 (pre-treatment). In chloroform-treated sheep, on an average, there was no significant affect in the relative abundance of members of the *Methanobrevibacter ruminantium* clade (p = 1.000) compared to day 1. Interestingly, the relative abundance of archaea affiliated with *Methanomassiliicoccales* Group 12 sp. ISO4-H5 increased significantly in acetylene-treated sheep over time by approximately 10-fold (p = 0.0001) as compared to day 1 and also increased significantly in chloroform-treated sheep (41.2%; p = 0.0001) from day 4 to day 5 although the affect was not significant at day 8 (p = 1.000) compared to day 1. The relative abundance of *Methanosphaera* sp. IS03-F5 decreased significantly (85.0%; p = 0.0099) in chloroform-treated sheep at day 8 as compared to day 1. Therefore, there were differences in archaeal community composition between acetylene- and chloroform-treated sheep, and these were large changes compared to the communities in the same sheep before dosing of the inhibitor commenced.

There were no significant differences (p = 1.000) in the relative abundances of different bacterial taxa in control sheep over time as they received no inhibitor (Figure 6.7a). In contrast to the stable total bacterial numbers in all sheep (Figure 6.5b and 6.5c), the bacterial community composition appeared to be affected in acetylene- and chloroform-treated sheep (Figure 6.7b and 6.7c). Overall, however, the changes were much smaller than they were in the archaea. In both inhibition treatments, the effects were most obvious in the days following the commencement

of treatment, but the communities seemed to be returning to their original structure after five days.

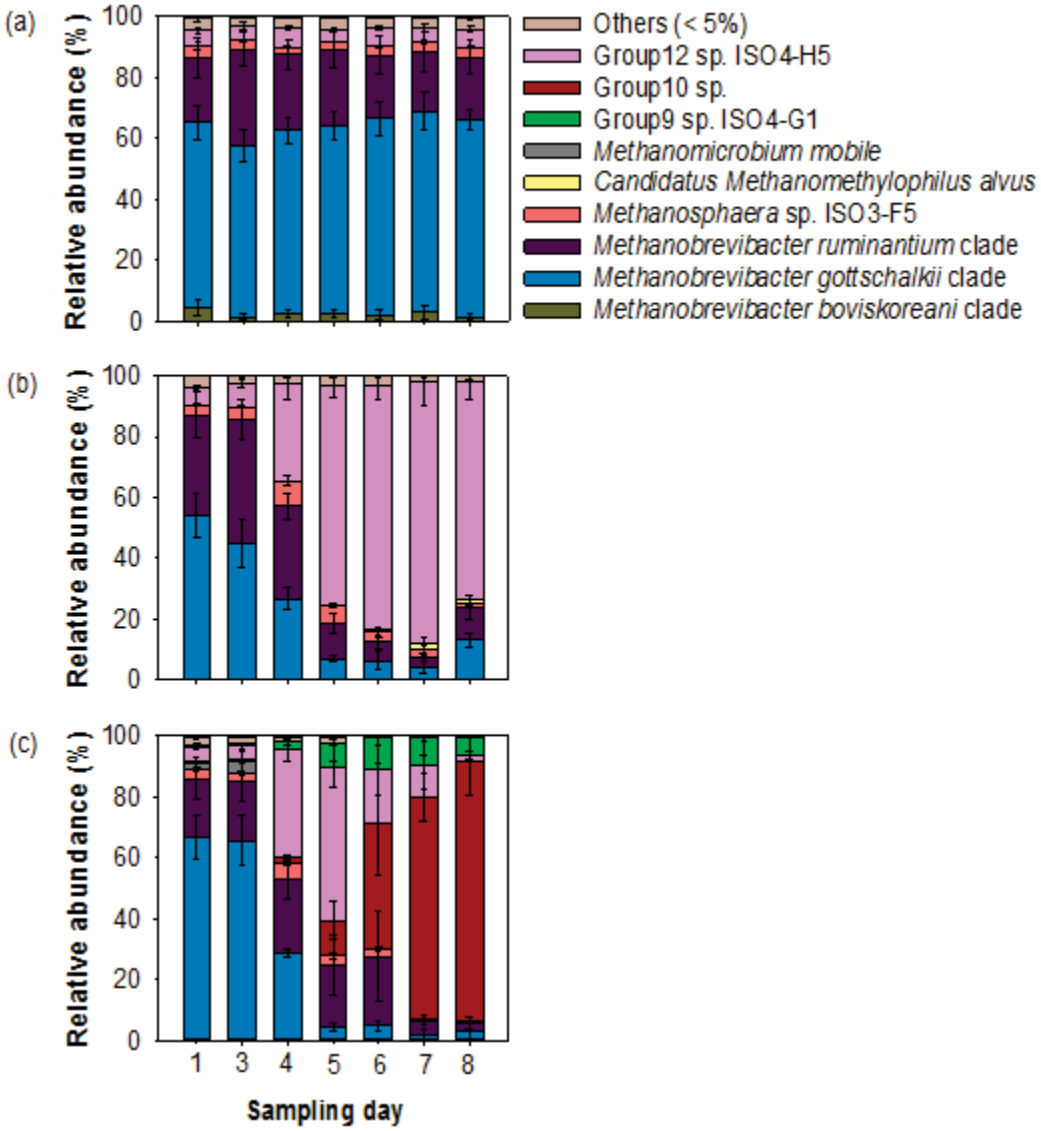

Figure 6.6 Archaeal community composition in rumen contents of sheep that received no inhibitor (a) and sheep that were dosed with acetylene (b) or chloroform (c). The data are from rumen samples collected at approximately 9 a.m. on the day noted. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, n = 4; chloroform-treated sheep, n = 3). Archaeal 16S rRNA gene sequences were classified at the species level. Group 9, 10 and 12 sp. belong to the order *Methanomassiliicoccales*. All taxa present at < 5% in all samples are grouped together as "Others" to simplfy the figure.

The relative abundance of unclassified members of the order *Clostridiales* (44.3%; p = 0.0002), and families *Lachnospiraceae* (47.3%; p = 0.0033) and *Ruminococcaceae* (36.5%; p = 0.0007) decreased significantly over time in acetylene-treated sheep as compared to day 1 (pre-treatment). The relative

abundance of *Prevotella* spp. increased significantly from day 4 to day 6 (39-64%; $p < 0.030$), but the increase was not significant on day 7 ($p = 0.152$) and day 8 ($p = 0.165$) compared to day 1 (pre-treatment). The relative abundance of *Fibrobacter* spp. had a trend to increase at day 4 (94.7%; $p = 0.087$), but there was no significant difference from day 5 to day 8 ($p = 1.000$) compared to day 1 (pre-treatment). The relative abundance of unclassified members of family *Veillonellaceae* ($p > 0.500$) and order *Bacteroidales* ($p > 0.100$) seemed to be unaffected over time compared to day 1 (pre-treatment). There was significant decrease in the relative abundance of *Ruminococcus* spp. on day 4 (67.8%; $p = 0.0001$), on day 5 there was trend to a decrease (58.9%; $p = 0.052$), but the difference was not significant on day 6 ($p = 0.165$) and day 7 ($p = 0.121$), although there was trend to a decrease on day 8 (60.7%; $p = 0.089$) again compared to day 1 (pre-treatment). The relative abundance of *Treponema s*pp. increased significantly from day 4 to day 6 (1.3 to 1.6-fold; $p < 0.05$), however there was no significant difference on day 7 ($p = 1.000$) and day 8 ($p = 1.000$) compared to day 1 (pre-treatment).

In chloroform-treated sheep, the relative abundance of unclassified members of the family *Ruminococcaceae* (54.8%; $p = 0.0001$) and the order *Clostridiales* (35.7%; $p < 0.04$) decreased significantly over time compared to day 1 (pre-treatment). The relative abundance of *Fibrobacter* spp. ($p = 1.000$), and unclassified members of family *Veillonellaceae* ($p = 1.000$) and order *Bacteroidales* ($p = 1.000$) seemed unaffected over time compared to day 1 (pre-treatment). The relative abundance of *Prevotella* spp. increased significantly from day 4 to day 7 (approximately 47% to 75%; $p < 0.002$), but the increase was not significant on day 8 ($p = 0.245$) compared to day 1 (pre-treatment). The relative abundance of *Ruminococcus* spp. decreased significantly from day 4 to day 7 (approximately 48% to 75%; $p < 0.005$), but the decrease was not significant on day 8 ($p = 0.117$) compared to day 1 (pre-treatment). There was no significant difference in the relative abundance of unclassified members of family *Lachnospiraceae* ($p > 0.700$), although there was significant decrease at day 5 (47.8%; $p = 0.0026$) compared to day 1 (pre-treatment). The relative abundance of *Treponema* spp. increased significantly from day 4 to day 8 (approximately 2 to 3-fold; $p < 0.020$), however there was no significant difference on day 6 but a trend towards increase (1.4-fold; $p = 0.072$) in the relative abundance compared to day 1 (pre-treatment).

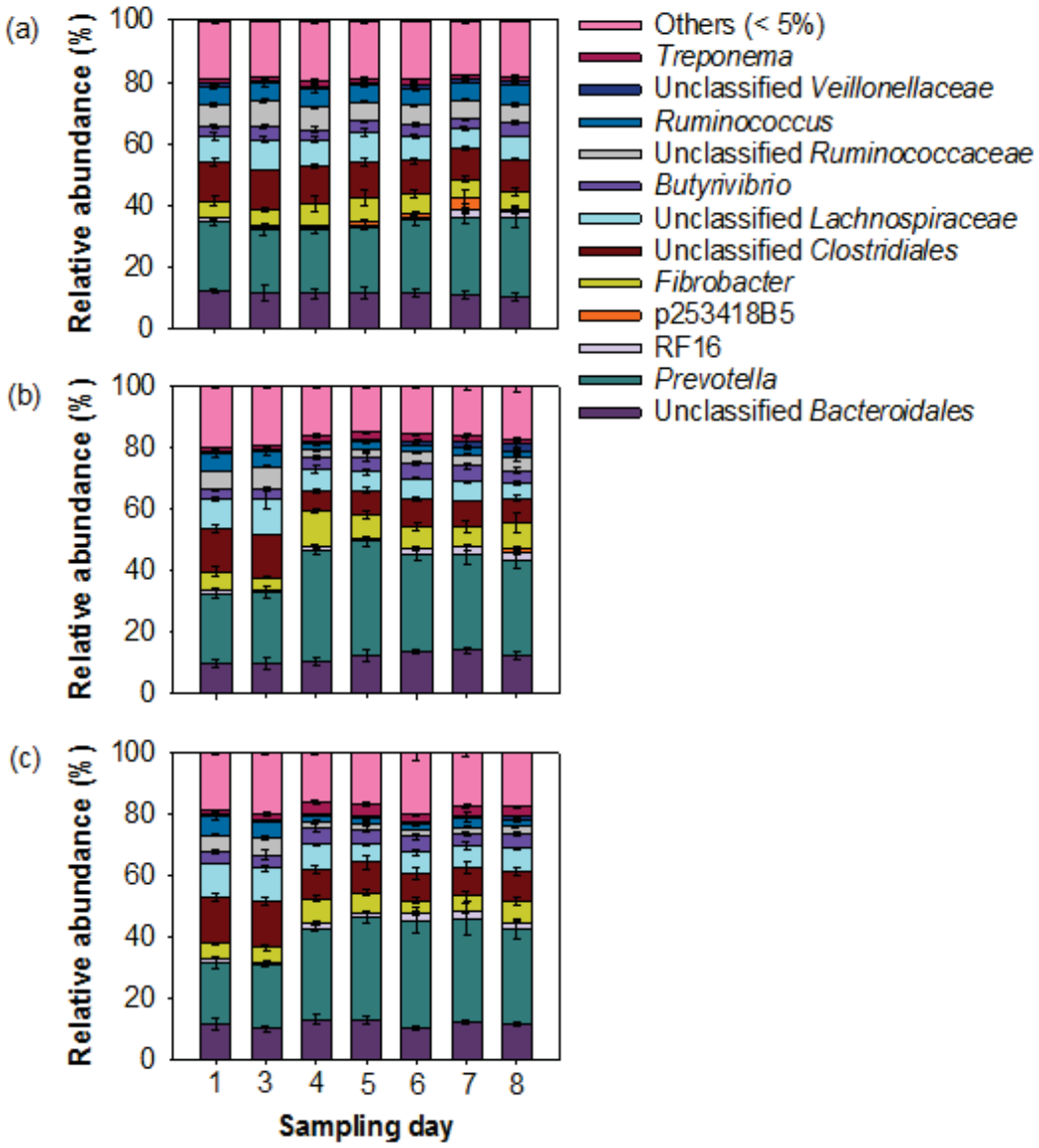

Figure 6.7 Bacterial community composition in rumen contents of sheep that received no inhibitor (a) and sheep that were dosed with acetylene (b) or chloroform (c). The data are from rumen samples collected at approximately 9 a.m. on the day noted. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, n = 4; chloroform-treated sheep, n = 3). Bacterial 16S rRNA gene sequences were classified at the genus level, except the groups containing sequences not able to be classified down to the genus level, which were represented as "unclassified" within the lowest ranked named taxon to which they belonged (family or order). All taxa present at < 5% in all samples are grouped together as "Others" to simplify the figure.

Principal component analysis (PCA) of bacterial community in control, acetylene- and chloroform-treated sheep over time showed that the communities changed with the administration of treatments (Figure 6.8a). Bacterial communities

in rumen contents of sheep that received no inhibitor showed that samples clustered together (Figure 6.8b). However, in acetylene- and chloroform-treated sheep the samples clustered apart from day 4 to 8 with the administration of treatment as compared to samples at day 1 and 3 (i.e., prior to treatment) and control sheep (Figure 6.8c and 6.8d). This indicated that acetylene and chloroform seemed to have effects on bacterial community structure in sheep but that these effects were different.

The most abundant and prevalent bacterial genus-level groups in the control, acetylene- and chloroform-treated sheep over time are provided in Appendix 3 (Table A.3.5. A.3.6 and A.3.7 respectively).

6.4.4 Identifying formyltetrahydrofolate synthetase sequences in sheep rumen samples using homoacetogen FTHFS profile hidden Markov model (HoF-HMM)

FTHFS gene sequencing was carried out on rumen samples from control (n = 4) and acetylene-treated (n = 4) sheep at day 1 (pre-treatment) and again at day 8 after five days of treatment with the inhibitor. FTHFS sequences (Table 6.2) from sheep rumen samples were scored against a homoacetogen FTHFS profile hidden Markov model (HoF-HMM), which was generated from FTHFS sequences from known homoacetogens. A HMMER bit score was assigned to each sequence based on its similarity with HoF-HMM with a corresponding E-value. The higher the bit score, the better the match with HoF-HMM. The HMMER bit scores of true FTHFS sequences obtained from GenBank tested against the HoF-HMM ranged from 409.1 to 791.2 and these were considered as significant matches. The formyltetrahydrofolate synthase, the enzyme encoded by this gene, can be involved in a number of pathways, and those from homoacetogens display distinctive structural features (Henderson *et al*. 2010). FTHFS sequences from known homoacetogens, which were used to develop the HoF-HMM had the best overall HoF-HMM bit scores, ranging from 732.8 to 791.2 and E-values ranging from 5.8e-121 to 5.3e-236.

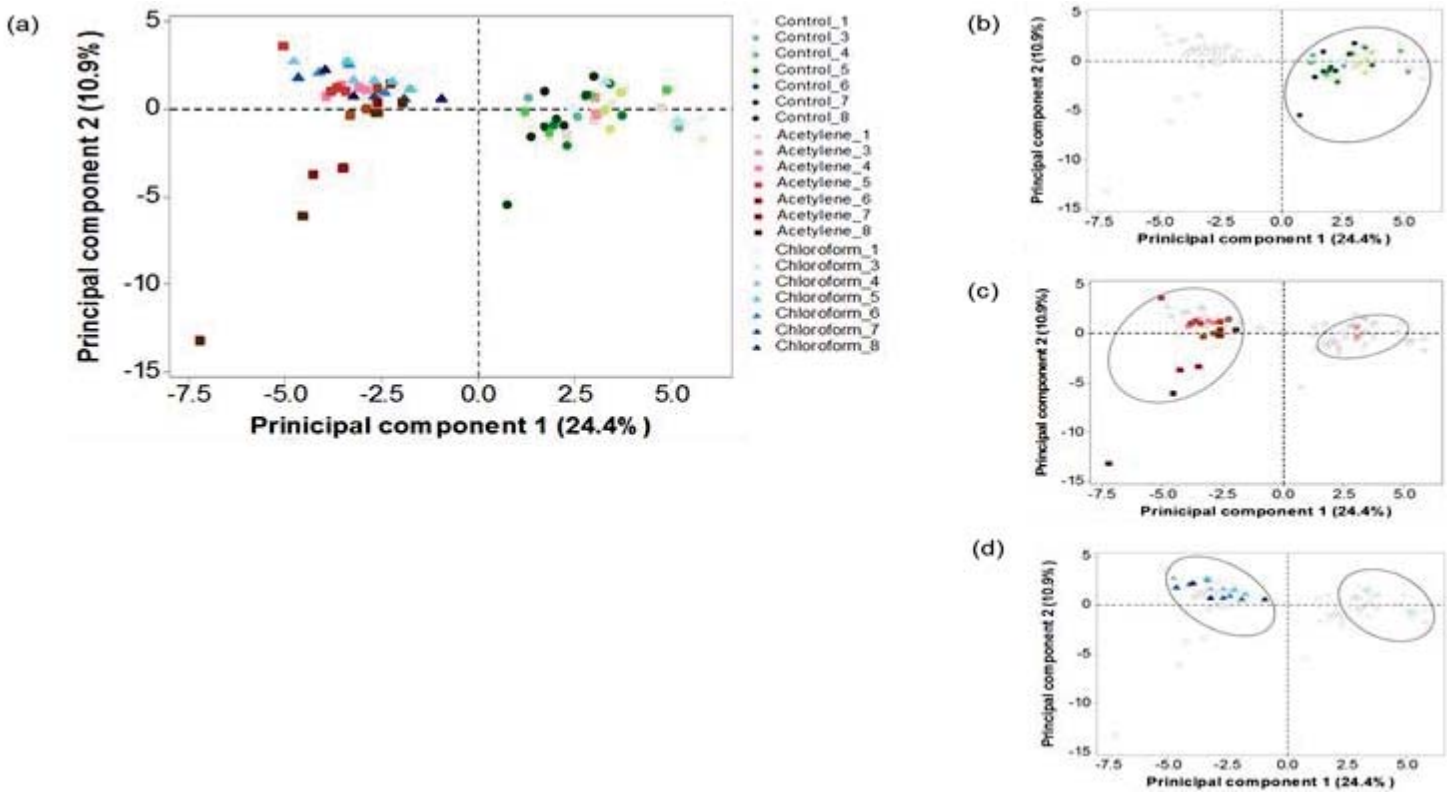

Figure 6.8 Scatter plot of principal component analysis (PCA) of bacterial communities in rumen contents of sheep that received no inhibitor and sheep that were dosed with acetylene or chloroform (a) over time. Data points highlighted in (b) represent bacterial communities in rumen contents of sheep that received no inhibitor and sheep that were dosed with acetylene (c) or chloroform (d) over time. The data are from rumen samples collected at approximately 9 a.m. on the day noted. The amounts of variation explained by principal component 1 and 2 are 24.4% and 10.9%% respectively. Panels (a), (b), (c) and (d) show the same data on the same axes. Open grey symbols present ponits not being considered in any one plot.

Therefore, FTHFS sequences that had a HMMER bit score of 700-800 were considered to be from homoacetogens, while those that had an intermediate HMMER bit score of 600-700 were considered less likely to be from homoacetogens. Sequences with a low HMMER bit score < 600 were probably from bacteria that were not homoacetogens.

Table 6.2. Number of FTHFS sequences analysed from each sheep. The numbers from the four sheep in each treatment group are listed in the same order by individual sheep for day 1 and day 8.

Inhibitor	Day 1	Day 8
None (n = 4[a])	45, 48, 55, 49	51, 55, 54, 54
Acetylene (n = 4[a])	44, 49, 49, 49	50, 51, 54, 51

[a]No. of sheep.

In the control and acetylene-treated sheep at day 1 (pre-treatment), 36.6% and 39.8% of FTHFS sequences had a high HMMER bit score, 48.6% and 30.6% an intermediate HMMER bit score and 14.8% and 29.6% had a low HMMER bit score (Figure 6.9). On day 8, during five days of treatment, control and acetylene-treated sheep had 35.2% and 40.0% FTHFS sequences with a high HMMER bit score, 37.8% and 35.7% with an intermediate HMMER bit score, and 27.0% and 24.2% with a low HMMER bit score. There were no significant differences in the HMMER bit scores of FTHFS sequences when methane production was inhibited in acetylene-treated sheep at day 8 ($p = 0.658$ to 0.755) and compared to the control sheep.

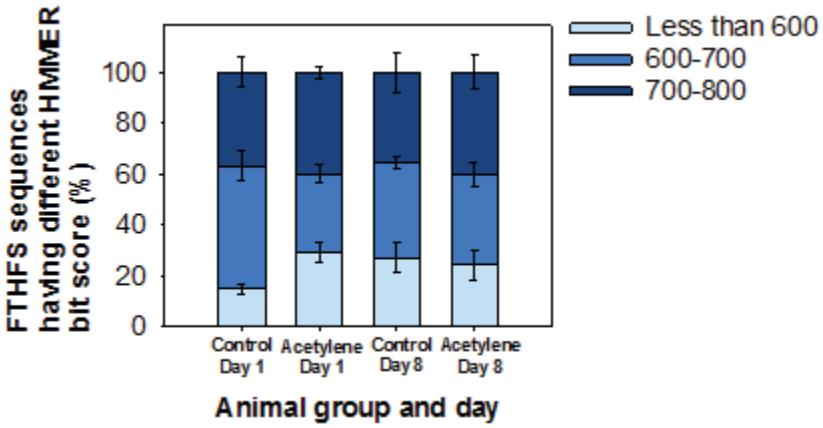

Figure 6.9 Similarity of FTHFS sequences from control and acetylene-treated sheep at day 1 (pre-treatment) and day 8 (during five days of dosing) to a Hidden Markov model containing FTHFS sequences from known homoacetogens. Scores were categorised as shown in the colour key. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, n = 4 in each group).

6.4.5 Measuring homoacetogenesis in sheep rumen contents using short-term *in vitro* homoacetogenesis assay

Rumen fluid was collected from sheep in the pre-treatment period (day 1) to study rumen homoacetogenesis and again during the treatment period (day 8) to study changes in homoacetogenic activity during five days of inhibition of methane production using acetylene or chloroform in sheep, using the short-term homoacetogenesis assay (Chapter 4). Various fermentation variables such as total gas, hydrogen, methane and VFA production were also assessed in these assays. This short-term *in vitro* assay represents the activity in the rumen at the time of sampling, and can be used to estimate activities like VFA formation that are confounded in the animal by uptake and passage from the rumen.

6.4.5.1 Inhibition of methane production *in vitro* with addition of inhibitors

In the pre-treatment period (day 1), the *in vitro* fermentation from all three groups of sheep, control (Figure 6.10a), acetylene-treated (Figure 6.10b) and chloroform-treated (Figure 6.10c), behaved the same. For example, using rumen fluid from the control sheep, BES and chloroform inhibited methane production significantly in the *in vitro* assays by 97.5% ($p < 0.0001$) and 98.9% ($p < 0.0001$) respectively as compared to the assay with no inhibitor. Hydrogen was also produced in the BES-supplemented assay (14.3% v/v of total gas; $p = 0.001$) and the chloroform-supplemented assay (14.6% v/v of total gas; $p < 0.0001$) as compared to the unamended assay (0.55% v/v of total gas). Total gas seemed to be unaffected by addition of BES ($p = 0.874$) or chloroform ($p = 0.700$).

During five days of treatment (day 8), rumen fluid from the control sheep (Figure 6.10d) behaved the same as in the pre-treatment period (Figure 6.10a). In acetylene-treated sheep, however, only a very small amount of methane was detected in the assay (< 0.001 mmol/bottle), as acetylene had inhibited methane production in these sheep (Figure 6.10e). Presumably, there were very few active methanogens in the rumen fluid added to the *in vitro* assay. Further addition of BES or chloroform to the *in vitro* assay virtually abolished all residual methane production by rumen fluid from the acetylene-treated sheep. Hydrogen was formed by rumen fluid from the acetylene-treated sheep (9.36% v/v of total gas), and was not significantly different in the BES- (7.01% v/v of total gas; $p = 0.563$) and

chloroform-amended assays (10.4% v/v of total gas; p = 0.905). Total gas formation by rumen fluid from acetylene-treated sheep was not affected by addition of BES (p = 0.927) or chloroform (p = 1.000) as compared to the assay without added inhibitor.

The amount of methane formed in the *in vitro* assay by rumen fluid from the chloroform-treated sheep in the treatment period was < 0.005 mmol/bottle, as chloroform inhibited methane production in the sheep (Figure 6.10f), and again had presumably eliminated nearly all the active methanogens. Addition of BES to the assay reduced methane formation completely by 97.7%. Hydrogen was formed by the rumen fluid (12.4% v/v of total gas), and this was not different when BES (12.9% v/v of total gas; p = 0.758) and chloroform (14.2% v/v of total gas; p = 0.332) were added. The total gas produced by the rumen fluid from the chloroform-dosed sheep also seemed to be unaffected by BES (p = 0.757) and chloroform (p = 0.590) addition as compared to the assay without added inhibitors.

6.4.5.2 Changes in volatile fatty acid profile of rumen fluid incubated *in vitro*

Acetate production by rumen fluid from the control sheep, collected in the pre-treatment period, decreased significantly (p < 0.0001) by 29.8% and propionate increased significantly (p = 0.007) by 35.8%, whereas butyrate formation did not change significantly (p = 0.821) in the *in vitro* incubations amended with BES compared to the assay with no additions (Figure 6.11a). Addition of chloroform to the assay had the same effects on acetate (decreased by 31.3%; p < 0.0001), propionate (increased by 44.5%; p = 0.002) and butyrate (no change; p = 0.868) as compared to the assay with no inhibitor. All the *in vitro* incubations of rumen fluid from all the three treatment groups (control, acetylene- and chloroform-treated sheep) behaved the same way in in the pre-inhibition period (Figure 6.11a, 6.11b and 6.11c), and rumen fluid from the control sheep behaved the same even at day 8 (Figure 6.11d).

The total amount of acetate produced was lower and more propionate was produced, with not much difference in butyrate when rumen contents collected from the acetylene-treated sheep during the treatment period (day 8) were incubated *in vitro*, compared to the control sheep (Figure 6.11e). What is striking is that addition

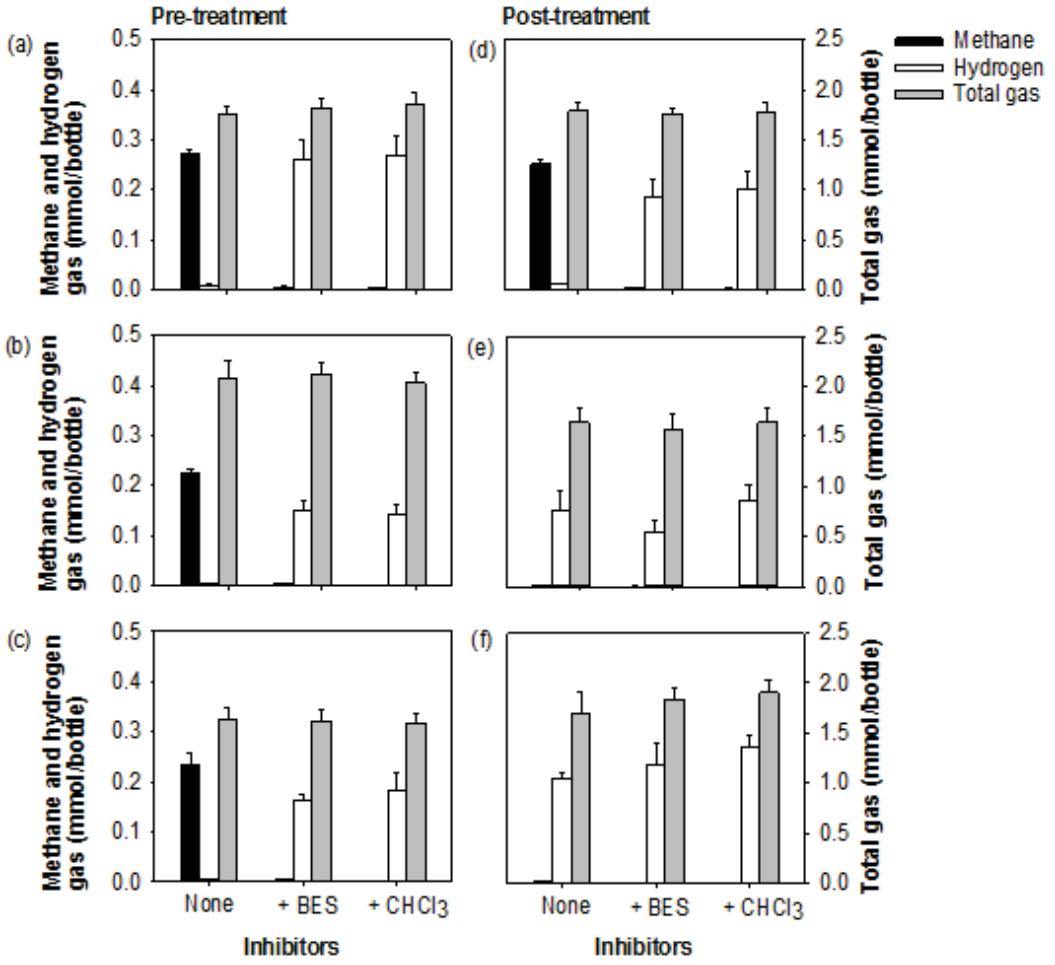

Figure 6.10 Methane, hydrogen and total gas produced by rumen contents incubated *in vitro* for 8 h. Panels on the left side show gases produced by rumen contents collected from control (a), acetylene-treated (b) and chloroform-treated (c) sheep in the pre-treatment period (day 1). Panels on the right side show gases produced by rumen contents collected from control (d), acetylene-treated (e) and chloroform-treated (f) sheep during the treatment period (day 8). The left and right axes apply to all panels. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, n = 4; chloroform-treated sheep, n = 3).

of BES (p = 0.966 to 0.981) or chloroform (p = 0.279 to 0.940) to these *in vitro* assays had no significant effect on VFA production. The same pattern was found with rumen contents from the chloroform-treated sheep on day 8. The total amount of acetate produced was decreased by approximately 30-35% and propionate increased by approximately 50-60%, with no major difference in butyrate, compared to the control sheep (Figure 6.11f), and there was no significant difference when BES (p = 0.995 to 0.999) or chloroform (p = 0.817 to 0.999) were added to the assay.

These *in vitro* assay results are also consistent with proportions of acetate, propionate and butyrate measured in the rumens of the sheep at day 8, i.e., there was a decrease in the proportion of acetate and an increase in the proportion of propionate with inhibition of methane in sheep (Figure 6.3). This suggests that the ruminal concentrations reflected the actual production rates of the different VFA in the short-term assay, if we accept that the samples continue to produce VFA when transferred to the *in vitro* assay. In the rumen, there are unknown rates of VFA production, absorption across the rumen wall, and also exit from the rumen into the lower digestive tract, all of which result in a measured concentration. In the *in vitro* assay, VFA accumulate, and production rates can be estimated.

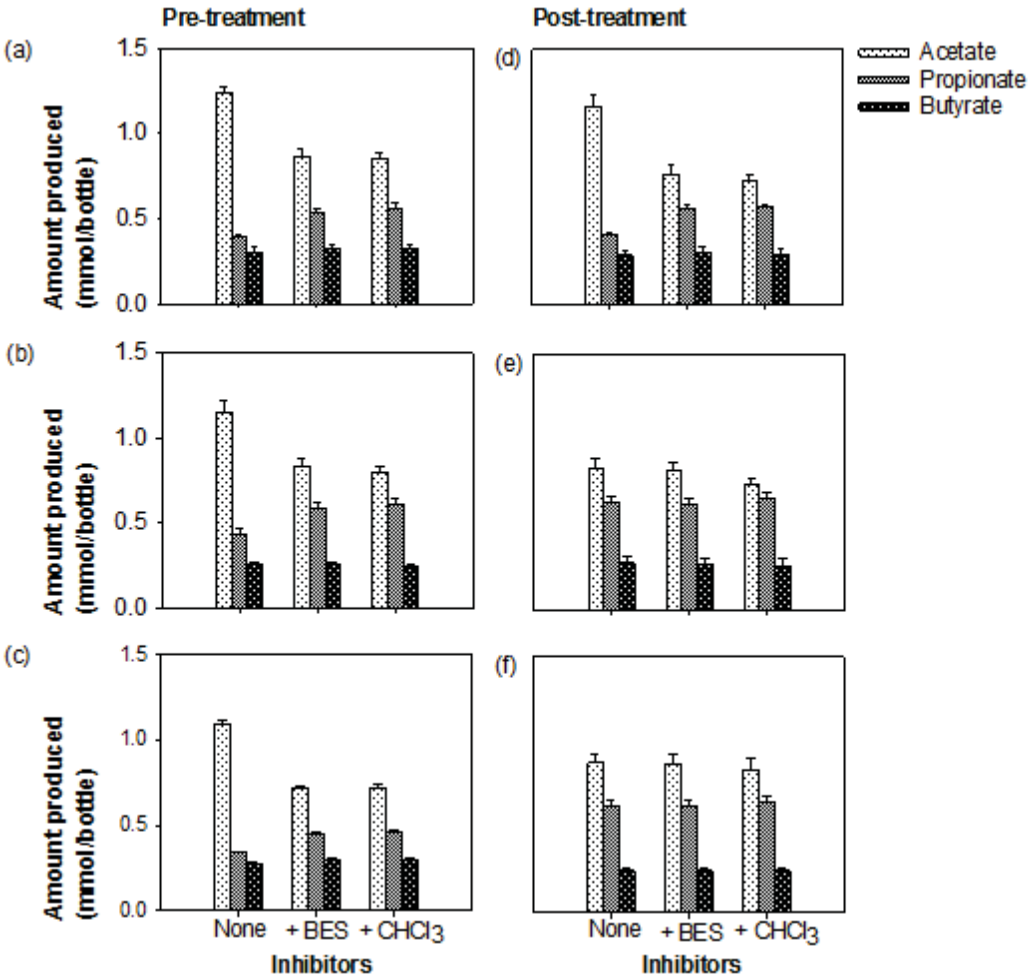

Figure 6.11 Volatile fatty acids produced by rumen contents incubated *in vitro* after 8 h. Panels on the left side depict VFA produced by rumen contents collected from control (a), acetylene-treated (b) and chloroform-treated (c) sheep in the pre-treatment period (day 1). Panels on the right side depict VFA produced by rumen contents collected from control (d), acetylene-treated (e) and chloroform-treated (f) sheep during the treatment period (day 8). The left axes apply to all panels. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).

6.4.5.3 Homoacetogenesis is present in sheep rumen fluid and increases during inhibition of methanogenesis over five days in sheep

To determine the amount of homoacetogenic activity in sheep rumen fluid before and during the treatment period, the incorporation of $^{13}CO_2$ into ^{13}C-acetate and other fermentation products was measured *in vitro* using the short-term (8 h) homoacetogenesis assay (Chapter 4). Homobutyrogenesis, the formation of butyrate from CO_2 and H_2, was also determined. The quantities of ^{13}C-labelled acetate produced via homoacetogenesis ($^{13}A_{ha}$) and ^{13}C-butyrate produced via homobutyrogenesis ($^{13}B_{hb}$) were calculated using data described in the Appendix 3 (Table A.3.8 and A.3.9), and were corrected for VFA inter-conversion by using the fractional amounts of VFA inter-conversion (equations 1 and 2) described in detail in Chapter 4 and Figure 4.14.

$$^{13}A_{ha} = {}^{13}A - f_{pa} \times {}^{13}P - f_{ba} \times {}^{13}B + f_{ap} \times {}^{13}A + f_{ab} \times {}^{13}A \quad (1)$$

$$^{13}B_{hb} = {}^{13}B - f_{pb} \times {}^{13}P - f_{ab} \times {}^{13}A + f_{bp} \times {}^{13}B + f_{ba} \times {}^{13}B \quad (2)$$

Finally, from the ratio excess of $^{13}CO_2$ measured using IRMS ($r^{13}CO_2$), the total quantities of acetate (unlabelled and labelled) produced via homoacetogenesis (A_{ha}) and the total quantity of butyrate (unlabelled and labelled) produced via homobutyrogenesis (B_{hb}) were calculated (equations 3 and 4).

$$A_{ha} = {}^{13}A_{ha}/r^{13}CO_2 \quad (3)$$

$$B_{hb} = {}^{13}B_{hb}/r^{13}CO_2 \quad (4)$$

The amounts of acetate formed from homoacetogenesis and butyrate from homobutyrogenesis were expressed as both the amount produced in μmol/bottle (Figure 6.12), and as percentages of total acetate ($A_{ha}/A \times 100$) and butyrate produced ($B_{ha}/B \times 100$; Figure 6.13).

The amount of acetate formed from homoacetogenesis (A_{ha}) in rumen fluid from the control sheep in the pre-treatment period (day 1) was 9.53 μmol/bottle and butyrate from homobutyrogenesis (B_{hb}) was 1.24 μmol/bottle (Figure 6.12a). Acetate from homoacetogenesis and butyrate from homobutyrogenesis was 13.5 μmol/bottle ($p = 0.155$) and 1.72 μmol/bottle ($p = 0.803$) respectively with the addition of BES to the assay, with no significant differences compared to the assay

with no inhibitor. In contrast, acetate from homoacetogenesis was dramatically reduced ($p = 0.009$) by 81.1% to 1.80 μmol/bottle, with no significant affect on butyrate from homobutyrogenesis (1.76 μmol/bottle; $p = 0.751$), with addition of chloroform as compared to the assay without added inhibitors. Homoacetogenesis and homobutyrogenesis seemed to follow a similar pattern in rumen fluid from all the sheep ($n = 12$) in the pre-treatment period (day 1; Figure 6.12a, 6.12b and 6.12c), and was similar in rumen fluid from the control sheep during the treatment period (day 8) as well (Figure 6.12d).

Homoacetogenesis and homobutyrogenesis increased in the acetylene-treated sheep during five days of dosing, compared to the control sheep. Using rumen contents collected from the control sheep on day 8, the amount of acetate formed from homoacetogenesis and butyrate from homobutyrogenesis was 10.6 and 0.82 μmol/bottle, respectively (Figure 6.12d). Using rumen contents from the acetylene-treated sheep, the amount of acetate formed from homoacetogenesis increased by 2.4-fold to 35.8 and butyrate from homobutyrogenesis by 52.4% to 1.24 μmol/bottle as compared to control sheep at day 8, and was unaffected ($p = 0.992$) by BES addition (Figure 6.12e). Similarly, the amount of acetate from homoacetogenesis and butyrate from homobutyrogenesis was 26.6 and 0.66 μmol/bottle from rumen contents from the chloroform-treated sheep (Figure 6.12f), and was unaffected ($p = 0.955$) by the addition of BES. Further addition of chloroform *in vitro* significantly decreased acetate from homoacetogenesis ($p < 0.0001$) by 90.5% to 2.53 μmol/bottle and increased butyrate from homobutyrogenesis ($p < 0.010$) by 49.8% to 0.95 μmol/bottle as compared to assays with no inhibitor. The lower amount of acetate formation from homoacetogenesis measured in the samples from the chloroform-treated sheep as compared to acetylene-treated sheep is probably due to inhibition of homoacetogens in sheep rumen, as obvious from previous studies as well. However, homoacetogenesis was reduced even more *in vitro* with supplementation of chloroform, although chloroform was administered 3 times (300 μM) of the amount added in the *in vitro* (100 μM). This might be because chloroform was washed out quickly in the rumen as compared to the *in vitro* system, and it was not continuously available to inhibit homoacetogens and eliminate them.

In summary, by measuring homoacetogenesis by incorporation of $^{13}CO_2$ into ^{13}C-acetate *in vitro*, it was shown that ruminal homoacetogenesis increased 2.75-fold during inhibition of methanogenesis during five days of treatment of sheep with acetylene as compared to control sheep at day 1.

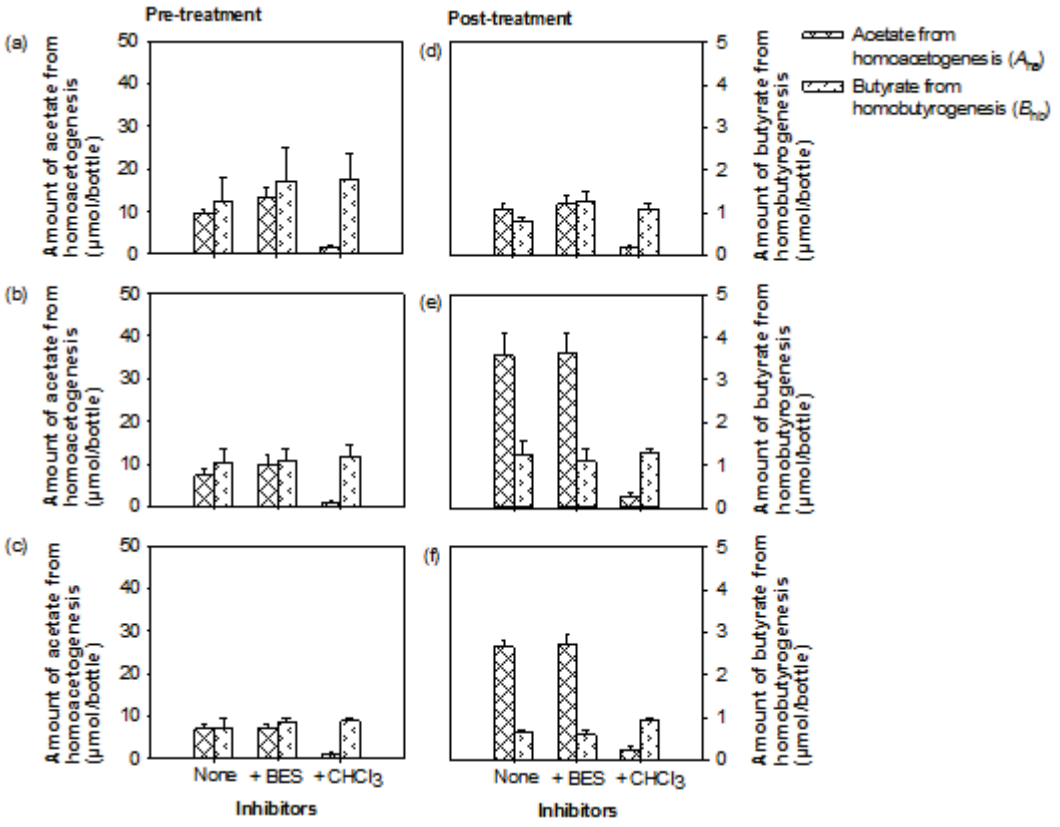

Figure 6.12 Amounts of acetate formed from homoacetogenesis (A_{ha}) and butyrate formed from homobutyrogenesis (B_{hb}) following incubation of sheep rumen contents *in vitro* for 8 h. Panels on the left side depict acetate and butyrate generated by homoacetogenesis and homobutyrogenesis respectively by rumen contents collected from control (a), acetylene-treated (b) and chloroform-treated (c) sheep in the pre-treatment period (day 1). Panels on the right side depict acetate and butyrate generated by homoacetogenesis and homobutyrogenesis respectively by rumen contents collected from control (d), acetylene-treated (e) and chloroform-treated (f) sheep during the treatment period (day 8). The left and right axes apply to all panels. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).

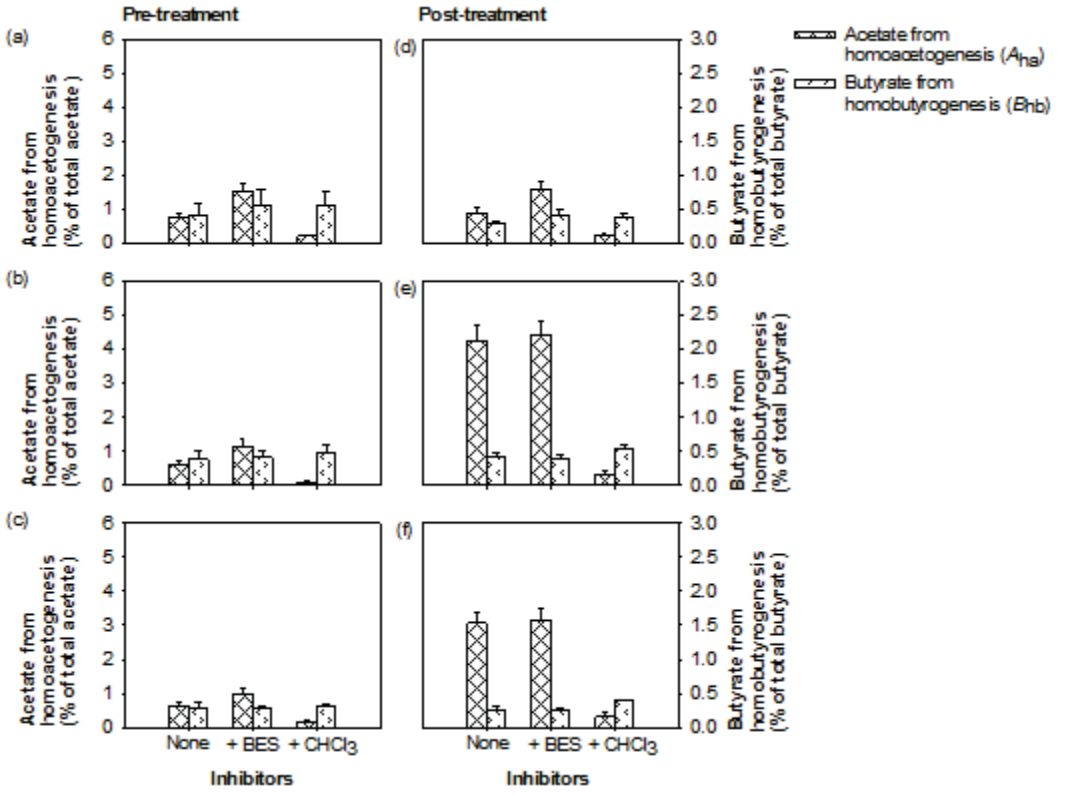

Figure 6.13 Percentage of total acetate produced from homoacetogenesis (A_{ha}) and total butyrate produced from homobutyrogenesis (B_{hb}) following incubation of rumen contents *in vitro* for 8 h. Panels on the left side depict acetate and butyrate generated by homoacetogenesis and homobutyrogenesis respectively by rumen contents collected from control (a), acetylene-treated (b) and chloroform-treated (c) sheep in the pre-treatment period (day 1). Panels on right side depict acetate and butyrate generated by homoacetogenesis and homobutyrogenesis respectively by rumen contents collected from control (d), acetylene-treated (e) and chloroform-treated (f) sheep during the treatment period (day 8). The left and right axes apply to all panels. Data are presented as means ± standard errors of the mean (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).

6.4.5.4 Hydrogen concentration versus relative rate of hydrogen metabolism for homoacetogens

The theoretical relative rate of hydrogen metabolism by homoacetogens versus the concentration of hydrogen was calculated as described in Chapter 4 (equation 21). In the incubations of rumen contents from the three groups of sheep collected in the pre-treatment period (day 1), the hydrogen concentration was less than 1.5 μM, and the calculated relative rate of metabolism of homoacetogens was also very small (< 0.002; Figure 6.14b). This is consistent with small amount of homoacetogenesis measured prior to treatment (Figure 6.12a, 6.12b and 6.12c). At

day 8 of the experiment, rumen contents from control sheep still resulted in small hydrogen concentrations, around 1.72 μM, and the calculated relative rate of metabolism of homoacetogens was correspondingly very small (< 0.002; Figure 6.14c). This again seems to be consistent with the small amount of acetate formed from homoacetogenesis at day 8 from control sheep (Figure 6.12d). After inhibition of methane using acetylene, the hydrogen concentration in *in vitro* assays using rumen fluid collected at day 8 increased by 16.8-fold to 30.6 μM and using rumen fluid from chloroform-treated sheep by 23.4-fold to 42.0 μM (Figure 6.14c) compared to rumen fluid from control sheep. Therefore, the calculated relative rates of metabolism of homoacetogens also increased by 20.7-fold and 28.4-fold, to 0.041 and 0.055 in acetylene- and chloroform-treated sheep rumen fluid respectively. This might therefore explain the increase in homoacetogenesis in acetylene- and chloroform-treated sheep rumen fluid at day 8 (Figure 6.12e and 6.12f). However, the increase in acetylene-treated sheep was greater by 25.5% than in chloroform-treated sheep, as chloroform inhibits homoacetogens.

These calculations suggest that the increased amount of homoacetogenesis in the *in vitro* assays could have been due to higher hydrogen concentrations in the sheep rumen when methanogens are inhibited with acetylene or chloroform. Increased hydrogen emissions from animals often reported when methanogens are inhibited (Mitsumori *et al.* 2012, Hristov *et al.* 2015), and the increased emissions can reasonably be expected to be associated with increased concentrations of dissolved hydrogen in the rumen. Of course, homoacetogens can also grow heterotrophically, where acetate formation from CO_2 via the Wood-Ljungdahl pathway is used as an electron-disposing mechanism. That activity would also be measured in the short-term assay, and would correctly be interpreted as homoacetogenesis, although not in response to hydrogen. Overall, however, it is reasonable to conclude that, when methanogens are inhibited in the sheep rumen, endogenous homoacetogenic activity increases, as has often been postulated (Boccazzi & Patterson 1996, Joblin 1999, Ungerfeld 2015).

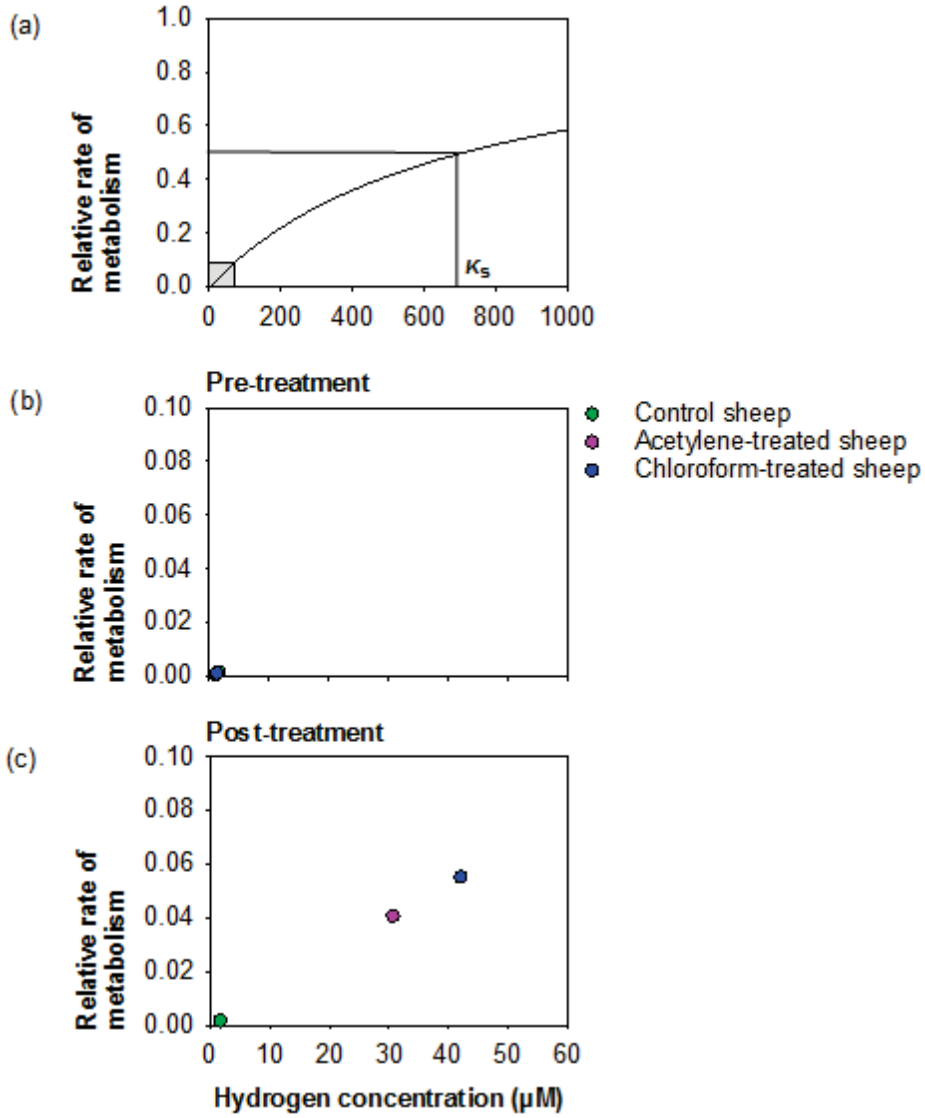

Figure 6.14 Theoretical relative rates of metabolism versus hydrogen concentration for homoacetogens (a) with K_s = 710 µM and S_{min} = 383 nM. The shaded area in (a) was then plotted using actual data points, i.e., hydrogen concentrations versus calculated relative rate of metabolism of homoacetogens in fresh sheep rumen contents collected from control, acetylene-treated and chloroform-treated sheep in the pre-treatment period and incubated *in vitro* with no additional inhibitors for 8 h (b), and again with rumen contents collected during the treatment period (day 8; c). Data are presented as means (control and acetylene sheep, n = 4; chloroform sheep, n = 3).

6.4.5.5 Electron balance during short-term *in vitro* rumen fermentation

The electron balance was calculated from data produced from the 8-h *in vitro* incubations of rumen fluid collected in the pre-treatment (day 1) and during the treatment (day 8) periods. The electron balance was calculated according to equations 5 and 6, described in Chapter 4.

$$2H \text{ produced} = 2A_f + P_f + 4B_f + 3V_f \quad (5)$$

$$2H \text{ utilised} = 2P_f + 2B_f + 4V_f + 4A_{ha} + 10B_{hb} + 4M + H_2 \quad (6)$$

6.4.5.5.1 Increase in electron utilisation by homoacetogenesis during inhibition of methane production

The proportion of electrons utilised by the formation of each metabolite, as a proportion of total electrons utilised, was calculated using data from the 8-h *in vitro* homoacetogenesis assay (Figure 6.15). In the pre-treatment period (day 1), the three groups of animals (control, acetylene-treated and chloroform-treated) had very similar electron utilisation profiles. On average, 40.4% of the *2H* was utilised in methane production, 32.5% in propionate, 22.9% in butyrate, and 2.05% in valerate production, 1.32% in homoacetogenesis, 0.41% in homobutyrogenesis, and 0.31% in the hydrogen produced (Figure 6.15a, 6.15b and 6.15c). Addition of BES to the *in vitro* assay resulted in, on average, a decrease of *2H* utilisation in methane formation to 1.21%, and increased utilisation in propionate formation to 53.4%, butyrate formation to 29.8%, valerate formation to 3.34%, hydrogen formation 9.62%, and more homoacetogenesis to 2.03%, and more homobutyrogenesis to 0.60%. Addition of chloroform *in vitro* had very similar effects to BES addition, except that *2H* utilisation by homoacetogenesis was reduced to 0.24%, as chloroform partially inhibits homoacetogenesis. It is clear that over 8 h of *in vitro* inhibition of methane production by BES, *2H* utilisation by homoacetogenesis increased on an average from 1.32% to 2.03% and *2H* utilisation by propionate formation increased from 32.5% to 53.4%. This showed that, as found in Chapter 4, that rumen fluid from normal sheep contained homoacetogens that these could increase their activity if methanogens were inhibited, and that this activity was sensitive to chloroform. The sheep studied in Chapter 4 had been fed pasture, while these were fed GP, a diet containing hay, soya, barley and molasses. So, homoacetogens appear to be present as a normal part of the microbiota of the sheep rumen.

When methanogenesis was inhibited *in vivo* over five days using acetylene or chloroform, there were changes in the electron balance as compared to the pre-treatment period and the control sheep (Figure 6.15e and 6.15f). The control sheep behaved the same in the pre-treatment and treatment periods (Figure 6.15a and 6.15d). In acetylene-treated sheep with inhibition of methane production, the

amount of *2H* utilised in homoacetogenesis increased to 6.53-6.91% and this was not stimulated further by BES addition (Figure 6.15e). This suggests that the homoacetogens were fully active, in contrast to the pre-treatment and control sheep. Addition of chloroform to the *in vitro* assay containing rumen fluid from the acetylene-treated sheep decreased the amount of *2H* utilised by homoacetogenesis to 0.49%. The major shift in the *2H* utilisation was towards propionate to 60.2%. In chloroform-treated sheep the amount of *2H* utilised in homoacetogenesis increased to 4.94-5.05% in the assay and was also not affected by BES (Figure 6.13f). Further addition of chloroform to the *in vitro* assay reduced *2H* utilised by homoacetogenesis to 0.47%, which is expected to be due to further inhibition of homoacetogenesis by chloroform *in vitro*. Similar to acetylene-treated sheep, the major electron sink in the rumen fluid from the chloroform-treated sheep was propionate formation, which accounted for 58.3% of *2H* utilisation. This increase in *2H* utilisation by propionate mirrors the results seen earlier in this study, where there was an increase in ruminal concentrations and proportions of propionate (Figure 6.3), and an increased relative abundance of possible propionate-forming bacteria from the genus *Prevotella* in the acetylene- and chloroform-treated sheep (Figure 6.7b and 6.7c).

It appears that short-term inhibition of methanogenesis (for five days) using acetylene in the sheep rumen resulted in an increase in the amount of *2H* utilised in homoacetogenesis from 1.22-1.47% to 6.53%. This homoacetogenesis was sensitive to chloroform both *in vivo* and *in vitro*. Homobutyrogenesis was also detected, but it did not increase or decrease much, and contributed less than 1% to the *2H* utilisation before and during treatment. Propionate formation, however, seems to be the major pathway utilising electrons when methanogenesis is inhibited by acetylene or chloroform, increasing from 30.1-36.9% to 57.6-58.1%.

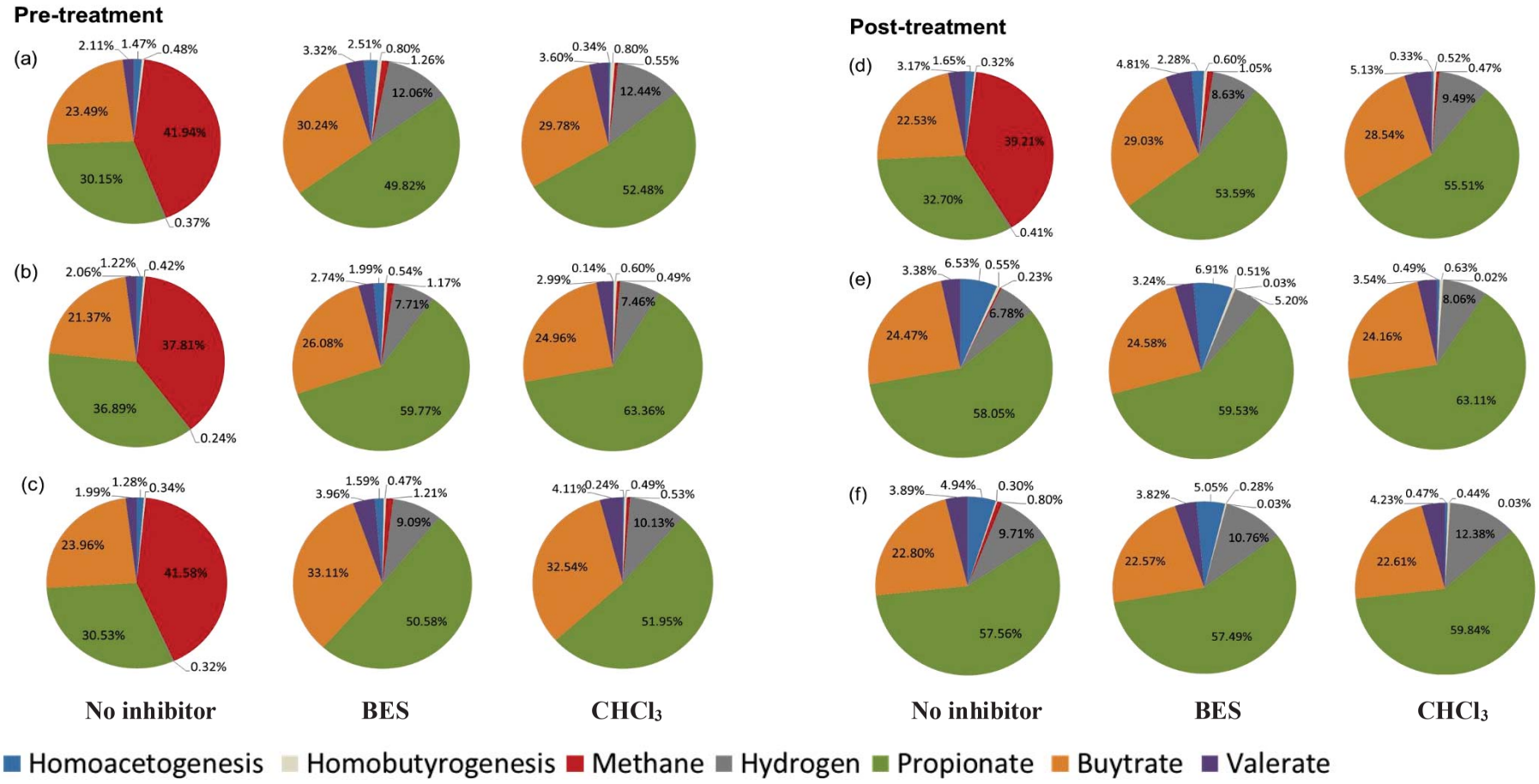

Figure 6.15 Electrons utilised (%) in production of various products in rumen contents incubated *in vitro* for 8 h, with different colours indicating the contribution of different sinks. Panels on the left side depict electrons utilised in rumen contents collected from control (a), acetylene-treated (b) and chloroform-treated (c) sheep in the pre-treatment period (day 1). Panels on the right side depict electrons utilised in rumen contents collected from control (d), acetylene-treated (e) and chloroform-treated (f) sheep during the treatment period (day 8). Data are presented as means (control and acetylene sheep, $n = 4$; chloroform sheep, $n = 3$).

6.4.5.5.2 Electron recovery

The electron recovery was calculated as the ratio between *2H* utilised (equation 6) and *2H* produced (equation 5) in percentage (equation 7):

2H % recovery = *2H* utilised × 100/*2H* produced (7)

The percentage of electron recovery in the pre-treatment and during the treatment periods was calculated for all the three groups of sheep (control, acetylene-treated and chloroform-treated), and summarised in Table 6.3. The percentage of electron recovery in all the three group of sheep in the pre-treatment period was 57.3-63.1%, regardless of the *in vitro* treatment (assay, BES or chloroform). During five days of inhibition of methane production (day 8), the percentage of electron recovery was only slightly greater, between 63.1-65.4% in all the three groups of sheep, regardless of *in vitro* treatment (assay, BES or chloroform).

Table 6.3 Electron recovery in sheep rumen contents incubated *in vitro* after 8 h in the pre-treatment period (day 1) and during the treatment period (day 8) period.

	Mean electron recovery (%, ±SEM[a]; *n* = 4 or 3[b])					
	Pre-treatment period and animal groups			**Post-treatment period and animal groups**		
Inhibitors added *in vitro* in short-term assay (8 h)	**Control sheep**	**Acetylene-treated sheep**	**Chloroform-treated sheep**	**Control sheep**	**Acetylene-treated sheep**	**Chloroform-treated sheep**
None	63.1 ± 0.68	62.0 ± 0.75	61.7 ± 1.89	65.1 ± 1.67	65.2 ± 1.83	64.2 ± 0.96
BES	59.5 ± 0.49	57.4 ± 0.95	56.9 ± 0.70	63.1 ± 1.74	64.3 ± 1.69	64.8 ± 0.59
$CHCl_3$	59.8 ± 0.82	57.3 ± 0.86	56.8 ± 0.39	63.5 ± 1.57	65.4 ± 1.63	64.5 ± 0.48

[a]Standard error of the mean.

[b]No. of sheep (control and acetylene-treated sheep, *n* = 4; chloroform-treated sheep, *n* = 3).

6.4.6 Discussion

Inhibition of methanogenesis in ruminants has been achieved using various inhibitors such as chloroform (Knight *et al.* 2011), bromochloromethane (Abecia *et al.* 2012, Mitsumori *et al.* 2012) and BES (Immig *et al.* 1996). An attempt was made to induce reductive acetogenesis in the rumen of a fistulated sheep using the methanogen-specific inhibitor BES but this failed, presumably due to the adaptation of methanogens to BES and methane emissions returned to control values after 4 days of BES infusion (Immig *et al.* 1996). Chloroform is a known inhibitor of methanogens and also inhibits homoacetogens (Prins & Lankhorst 1977, Conrad & Klose 2000, Scholten *et al.* 2000, Xu *et al.* 2010), and bromochloromethane may also inhibit homoacetogens because of its structural similarity to chloroform, so these might not be suitable inhibitors for studying the role of homoacetogens in the absence of methane formation. Therefore, acetylene was used as a specific-inhibitor of methanogenesis in this study. The impact of acetylene on methane production from sheep has also been previously assessed and it has been shown to inhibit methanogenesis by 100% for at least a week (M. Tavendale, personal communication). In the present study, there was a 98.7% reduction in methane emissions from sheep when the inhibitor was dosed in the rumen at approximately 3 mM dissolved acetylene. However, the amount of diet refused by the sheep increased during the five days of treatment with acetylene, and it cannot be considered for longer term inhibition of methane production. Chloroform was also used as an additional treatment because it is known to partially inhibit homoacetogenesis. Chloroform administered at a ruminal concentration of approximately 300 μM inhibited methane production significantly from sheep by 91.0%. This is similar to the inhibition reported by others. In sheep maintained on ration of lucerne and oaten chaff, chloroform inhibited methane formation over a period of 22 h at a concentration of 250 μM, (Lanigan 1972). Chloroform at a concentration of 200 μM was reported to inhibit methane production by 94-95% within 4-5 days in cattle fed a lucerne silage:concentrate diet (Knight *et al.* 2011).

There was a significant decrease in ruminal acetate, and immediate increases in ruminal propionate concentrations as a response to acetylene and chloroform dosing from day 4 to day 7 as compared to day 1, but these changes were not significant at day 8. The ruminal butyrate concentration seemed unaffected during

five days of treatment. However, acetate decreased proportionally and propionate remained proportionally more abundant as products during acetylene and chloroform treatment. Van Nevel *et al.* (1969) demonstrated that the inhibition of methanogenesis by chloral hydrate, which is converted into chloroform in the rumen contents, resulted in a 1.6-fold increase in propionate, and a decrease in acetate by 1.4-fold over five days, while butyrate increased by 1.3-fold (Van Nevel *et al.* 1969). Shifts in propionate and acetate concentrations were also reported in cow rumens dosed with 200 μM chloroform (Knight *et al.* 2011). This shift in rumen fermentation towards more propionate and less acetate proportion has been observed in other studies with methanogens inhibitors, and is thought to be due to a shift in reducing equivalents towards propionate formation, resulting in less acetate formation (Van Nevel *et al.* 1974, Nevel & Demeyer 2007). Decreases in methane production are strongly related to increases in propionate production (Janssen 2010). Other short-chain fatty acids (isobutyrate, isovalerate, valerate and caproate) were also measured in control, acetylene- and chloroform-treated sheep. However, these were not present in large amounts and did not differ significantly in the inhibitor treated groups compared to the control sheep. Lactate, formate and ethanol were also measured, but besides an increase in formate in chloroform-treated sheep at day 8, no other significant differences were measured. Since methane formation was still inhibited, it seems possible that the changes in ruminal concentrations of the propionate and acetate, back to concentrations similar to day 1 (pre-treatment), may be the result of changes in the rates of uptake across the rumen wall. It has been observed that increasing concentrations of acetate, propionate and butyrate in lambs rumen for 7.5 h, resulted in increases in the absorption rates of propionate and butyrate, by 46% and 33% respectively (Lopez *et al.* 2003). The changes in dry matter intake (DMI) and amounts of refusals that were observed in acetylene-treated sheep might also have caused some changes in the ruminal proportions of propionate and acetate, and probably explains the drop in ruminal proportion of acetate as acetylene-treatment progressed. However, in case of chloroform-treated sheep there were no refusals, but there were still changes in ruminal concentrations of propionate and acetate, back to that of day 1 (pre-treatment). This suggests that refusals did not explain all of the changes in ruminal concentrations of propionate and acetate, and that the change in absorption rates may also have played a role.

Inhibition of methane production using acetylene or chloroform in sheep resulted in a marked drop in total archaeal numbers, by more than 10-fold. This is consistent with more than 10-fold decrease in methane emissions. A similar drop in total archaeal numbers during chloroform treatment has been observed in the cattle rumen, where total archaeal numbers were quantified using a cultivation-dependent most-probable-number enumeration technique and decreased by 100-fold in 7 days as compared to their numbers prior to chloroform treatment (Knight *et al.* 2011). There were no significant effects of acetylene and chloroform on total bacterial numbers. The dramatic shifts in archaeal community structure were observed in both acetylene- and chloroform-treated sheep. *Methanobrevibacter* spp. were the most dominant methanogens in the rumen of control sheep, and this result is consistent with previous studies (Jeyanathan *et al.* 2011, Henderson *et al*. 2015, Seedorf *et al*. 2015). In studying the effect of chloroform in cattle rumens, it was observed by denaturing gradient gel electrophoretic analysis and clone libraries of 16S rRNA genes that the proportions of *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* in the methanogen community were greatly reduced, while the relative abundance of members of the *Methanomassiliicoccales* increased (Knight *et al.* 2011). The present results seem to be consistent with those results. There was a dramatic decrease in the relative abundance of 16S rRNA genes assigned to the *Methanobrevibacter gottschalkii* clade in both acetylene- and chloroform-treated sheep at day 8 as compared to day 1 (pre-treatment). In contrast, the relative abundance of members of the *Methanobrevibacter ruminantium* clade decreased in acetylene-treated sheep from day 6 to 7, although it was no longer significantly different by day 8 as compared to day 1 (pre-treatment). In chloroform-treated sheep, on an average, there was no significant affect in the relative abundance of members of the *Methanobrevibacter ruminantium* clade. Due to variations between individual animals, the difference was not statistically significant. At the same time, the relative abundance of *Methanosphaera* sp. ISO3-F5 increased in chloroform-treated sheep. Interestingly, the relative abundance of archaea affiliated with *Methanomassiliicoccales* Group 12 sp. ISO4-H5 increased in acetylene-treated sheep. In pure cultures studies (Chapter 3), it was found that *Methanomassiliicoccales* isolate ISO4-H5 was the least sensitive to acetylene, which might be the reason behind the increase in the relative abundance of this group of archaea in acetylene-treated sheep.

Members of the phyla *Bacteroidetes* (unclassified members of the order *Bacteroidales*, *Prevotella*, the RF16 group, and the P-2534 group) and *Firmicutes* (unclassified members of the order *Clostridriales*, unclassified members of the families *Lachnospiraceae*, *Ruminococcaceae* and *Veillonellaceae,* and genera *Butyrivibrio and Ruminococcus*) formed the major part of the bacterial community, as described in previous studies (Kim *et al.* 2011, Zened *et al.* 2013, Creevey *et al.* 2014, Danielsson *et al.* 2014, Henderson *et al.* 2015). Mitsumori *et al.* (2012) reported that treating goats with the methane inhibitor bromochloromethane (BCM) decreased the relative abundance of *Ruminococcus albus* (*Firmicutes*), and that there was an increase in the relative abundance of *Fibrobacter* spp. and *Prevotella* spp. (Mitsumori *et al.* 2012). *Prevotella* and *Fibrobacter* are known to be producers of propionate or the propionate precursor succinate (Bryant & Doetsch 1954, Dehority 1966, Howlett *et al.* 1976), and the changes in the bacterial community seem to compliment the observed changes in VFA production. The relative abundance of *Prevotella* spp. increased after treatment commenced, then started to decline again so that by days 7 and 8 the difference was no longer significant in acetylene- and chloroform-treated sheep. *Fibrobacter* spp. similarly showed a trend to increase but then decreased again by day 8. Therefore, the initial increase and then decline in ruminal propionate concentrations may be due to a changes in the populations invoved in its formation. However, propionate remained proportionally more abundant throughout acetylene and chloroform treatment. *Ruminococcus* spp. decreased after treatment but then increased again by day 8. These changes suggest that the major taxa in the rumen bacterial community were adapting to the change in methane formation after an initial shock, and appeared to be returning to a composition similar to the starting one, albeit with different products and less methane being formed.

In the past, studies have been carried out to study FTHFS encoded by the *fhs* gene as a marker for homoacetogens in various environments, such as the proximal and mid colon of the ostrich (Matsui *et al.* 2011), the gut of giant pandas (Tun *et al.* 2014), termite gut contents (Salmassi & Leadbetter 2003, Pester & Brune 2006), tammar wallaby fore-stomach and bovine rumen contents (Gagen *et al.* 2010, Henderson *et al.* 2010). In the present study, FTHFS sequences (actually translated partial sequences of PCR-amplified *fhs* genes) present in the sheep rumen samples were analysed using a homoacetogen FTHFS profile hidden Markov model

(HoF-HMM; Henderson *et al.* 2010). Because the enzyme has functions other than in homoacetogenesis, its presence alone cannot be used as a diagnostic marker of homoacetogens. However, the amino acid sequences of FTHFS variants involved in homoacetogenesis can be distinguished based on diagnostic amino acid residues. This allows each sequence to be given a score (HMMER bit score obtained with the FTHFS profile HMM, HoF-HMM, developed by Henderson *et al*. (2010)). In the control and acetylene-treated group of sheep in the pre-treatment period (day 1), 36% to 40% of FTHFS sequences had a high HMMER bit score and 30 to 49% has an intermediate HMMER bit score. During five days of inhibition of methane production in acetylene-treated sheep (day 8), there was no increase in FTHFS sequences with high or intermediate HMMER bit score (35% to 40% and 35% to 37% of all sequences, respectively). There may be some unknown homoacetogens with very different FTHFS that were not included in the reference HoF-HMM model, but the conclusion appears to be that there was not a large proliferation of homoacetogens in the rumen of the acetylene-treated sheep.

Homoacetogenesis was measured using a short-term *in vitro* homoacetogenesis assay before (day 1) and during the treatment period (day 8). In the pre-treatment period, the amount of acetate formed by homoacetogenesis was 7.14 to 9.53 μmol/bottle for rumen fluid from all sheep. When BES was added, this increased, to between 7.09 and 13.5 μmol/bottle for rumen fluid from all sheep, indicating that the homoacetogens had some capacity to increase their rate of metabolism. This was presumably not possible when methanogens were active, but inhibition of methanogens in the in *vitro* assay with BES resulted in an increased in hydrogen concentrations, allowing homoacetogens to increase their rates of metabolism. When rumen fluid from sheep after five days of inhibition of methane production using acetylene was used (day 8), acetate from homoacetogenesis significantly increased to 35.8 μmol/bottle, and using rumen fluid from chloroform-treated sheep it increased to 26.6 μmol/bottle. Adding BES to these assays did not significantly increase acetate formation by homoacetogenesis and acetate from homoacetogenesis was to 36.4 μmol/bottle using rumen fluid from acetylene-treated sheep, and 27.2 μmol/bottle in rumen fluid from chloroform-treated sheep. This implies that adding a methane inhibitor to the 8-h assay did not further increase homoacetogenesis, and this was the maximum potential for homoacetogenesis to increase. As also observed by calculating the dissolved hydrogen concentration and

rate of metabolism for homoacetogens in the *in vitro* assay, the hydrogen concentration was low and therefore the rate of metabolism for homoacetogens was low in rumen fluid from sheep in the pre-treatment period and the control sheep in the treatment (day 8). In assays using rumen fluid from the acetylene- and chloroform-treated sheep, the hydrogen concentration increased, thereby increasing the expected relative rate of metabolism for homoacetogens, and an actual increase in homoacetogenesis was measured. The response of homoacetogenesis to inhibition of methane production was less in the chloroform-treated sheep than in the acetylene-treated sheep, and homoacetogenesis in the assays was sensitive to further addition of chloroform, consistent with its known ability to partially inhibit homoacetogenesis. Butyrogenesis was also detected in the sheep, it increased by 22.4% in acetylene-treated sheep and decreased by 13.6% in chloroform-treated sheep at day 8 as compared to day 1.

Electron balances were calculated to study the utilisation of electrons (or metabolic hydrogen) by homoacetogenesis and other fermentation products. It was estimated that after five days of inhibition of methane production using acetylene, electron utilisation by homoacetogenesis increased from a maximum of 1.47% in the pre-treatment period (day 1) to a maximum of 6.53% in acetylene-treated sheep. There was therefore, a 4.44-fold increase in electron utilisation by homoacetogenesis. This suggests that homoacetogenesis makes a measurable contribution to the hydrogen/electron balance.

Propionate represented a major electron sink (Moss *et al.* 2000, Kobayashi 2010, Ungerfeld 2015) in the absence of methanogenesis, and this is in agreement with the increase in the proportion of propionate as compared to other products in the *in vitro* fermentations. Propionate formation represented 30.1-36.9% of the calculated electron sink in the sheep prior to treatment in all the sheep (in assays with no inhibitor), but during five days of inhibition with acetylene and chloroform, it accounted for 57.6-58.1%. This was also observed in a number of experiments conducted by others, where inhibition of methane production resulted in an increase in electron use for propionate formation with two contrasting diets and a series of additives during *in vitro* rumen fermentation (O'Brien *et al.* 2014, Ungerfeld 2015). The increase in electron utilisation by propionate by 1.78-fold in short-term *in vitro* assays using rumen contents from acetylene-treated sheep and 1.76-fold using

rumen contents from chloroform-treated sheep mirrors the increase in the proportion of propionate in the actual rumens of acetylene- (1.59-fold) and chloroform- (1.70-fold) treated sheep as compared to control sheep at day 8. Butyrate represented 21.4-23.9% of the electron total sink prior to inhibition in all the sheep, but after five days of inhibition of methane production with acetylene it increased to 24.5% and with chloroform to 22.8%. Therefore, there was not much increase or decrease in electron utilisation by butyrate. If we accept that the short-term *in vitro* assays represent the activity in the rumen at the time of sampling, this suggests propionate increased as an electron sink when methane formation was inhibited, and butyrate did not. This is consistent with the finding that propionate became proportionally more important as a VFA in the rumens of sheep treated with acetylene and chloroform. Hydrogen was insignificant in the pre-treatment period in assays without inhibitor, where it represented less than 0.4% of the total electron sinks, increased to 6.78% in short-term assays using rumen contents from acetylene-treated sheep and 9.7% using contents from chloroform-treated sheep. Hydrogen therefore, represented as significant electron sink during inhibition of methane production in sheep.

6.5 Summary and perspectives

The results from this sheep trial showed that the inhibitors acetylene or chloroform reduced methane production completely in sheep rumen over five days of treatment. Acetylene and chloroformdecreased total archaeal numbers and led to changes in archaeal community composition. There were no major effects on total bacterial numbers, but there were changes in the bacterial communities in both acetylene- and chloroform-treated sheep. The fermentation also shifted towards more ruminal propionate and less acetate as proportions of total VFA.

The short-term homoacetogenesis assay on sheep rumen fluid collected in the pre-treatment period (day 1) again confirmed that homoacetogenesis occurs in the sheep rumen. With the inhibition of methanogenesis in sheep by acetylene, acetate formation due to homoacetogenesis increased by 3.75-fold, and its significance as an electron sink increased 4.44-fold. A recent report on homoacetogenesis in the bovine rumen detected homoacetogenic activity using RNA stable isotope probing (RNA-SIP), but failed to quantify it (Godwin *et al.* 2014). There appeared to be no increase in the abundance of FTHFS sequences

homologous to known homoacetogens in the study reported in this Chapter, suggesting that the resident population became more active, but did not increase over five days. It is clear, however, that ruminal populations can respond very rapidly to perturbations in the rumen. Both the bacterial and archaeal communities changed within one day of the commencement of treatment with acetylene or chloroform.

When rumen methanogenesis was inhibited in sheep for five days using acetylene, the proportion of electron utilisation by homoacetogenesis increased 4.44-fold in the post-treatment period (day 8). It is not known if this is homoacetogenesis through the use of H_2 plus CO_2, or homoacetogenesis associated with heterotrophic fermentation by homoacetogens. Homoacetogenesis accounted for about 6.53% of electron utilisation when methane formation was inhibited with acetylene, and homoacetogenesis and homobutyrogenesis together accounted for over 7.08%, which was more than accounted for by net hydrogen formation, and was third in significance after propionate and butyrate formation. In the sheep with normal methane formation, homoacetogenesis and homobutyrogenesis together accounted for only 2%, and was less important than methane, propionate, butyrate, and valerate formation. This suggests that homoacetogenesis is not an insignificant hydrogen/electron sink when methane formation is inhibited.

The magnitude of homoacetogenesis has implications for hydrogen and electron balancing in the rumen. Using normal formal electron (metabolic hydrogen) balancing equations, this acetate would be expected to be associated with hydrogen production (two H_2 formed per acetate), but is actually a hydrogen sink (four H_2 used or saved per acetate). In addition, it inflates the acetate to propionate ratio, discounting the actual impact of propionate formation as an electron sink.

Chapter 7

Summary, conclusions and future recommendations

7.1 Summary and conclusions

Agriculture has always been important to New Zealand in terms of economy, employment and for its international exports. Besides its contribution to economy, agriculture contributes significantly to New Zealand's anthropogenic greenhouse gas emissions, with 47% of total greenhouse gas emissions coming from the agriculture sector (Ministry for the Environment, 2014). In New Zealand, 32% of total anthropogenic greenhouse gas emissions can be attributed to methane produced by ruminants (Ministry for the Environment, 2014) and globally methane emissions from enteric fermentation represent 27% of anthropogenic methane emissions (IPCC 2014b). This methane production contributes to the accumulation of GHGs in the atmosphere, which leads to changes in global climate such as an increase in global surface temperature (Moss *et al.* 2000, Gerstengarbe & Werner 2008). This further leads to melting of ice and glaciers, and changes in precipitation in low and high rainfall areas. Therefore, there is an interest in reducing methane emissions from ruminants in order to reduce ruminant agriculture's contribution to climate change. As well as, reducing methane emissions from ruminants may increase the productivity of the animals, as methane also represents a loss of 2-12% of the extractable energy in the feed consumed by the animals (Johnson & Johnson 1995).

A number of strategies have been developed to mitigate methane production from ruminants (Lascano & Cárdenas 2010, Knapp *et al.* 2014, Kumar *et al.* 2014), and these strategies still need to be improved to make them more practical and cost-effective. One of the most attractive of the various strategies being developed is direct inhibition of the methanogens, the microbes that are responsible for methane production. This could be achieved by developing specific chemical inhibitors (Bauchop 1967, Martin & Macy 1985, Dumitru *et al.* 2003, Hristov *et al.* 2015) or vaccines (Williams *et al.* 2009, Wedlock *et al.* 2013). Inhibiting methane formation alone might not increase the energetic efficiency of the animal, as inhibition of methane production might lead to accumulation of hydrogen in the rumen, which has been postulated to slow down feed fermentation due to accumulation of

reducing equivalents (McAllister & Newbold 2008). Therefore, hydrogen or electrons that are not utilised in methane production must be re-directed to some useful alternative hydrogen utilisation pathways or electron sinks present in the rumen. One such pathway is homoacetogenesis. Homoacetogens, the bacteria responsible for this process, are known to be present in the rumen (Leedle & Greening 1988, Henderson *et al.* 2010). Homoacetogens can use H_2 and CO_2 to form acetate, which is a source of energy for the animal, but in the presence of methanogens they are unable to compete for hydrogen (Joblin 1999, McAllister & Newbold 2008). A few previous studies have explored the potential of homoacetogens to utilise hydrogen, either by addition of homoacetogens in the absence of methanogens, or in the presence of feed additives, or by adding excess hydrogen *in vitro* (Chaucheyras *et al.* 1995, Nollet *et al.* 1997, Le Van *et al.* 1998, Nollet *et al.* 1998, Lopez *et al.* 1999). Some of these studies showed increases in acetate formation, but it was not clear whether it was from homoacetogenesis or carbohydrate fermentation. Homoacetogens are known to dominate in the developing rumen of lambs, but they are subsequently displaced by methanogens (Morvan *et al.* 1994, Fonty *et al.* 2007). Therefore, all these *in vitro* and *in vivo* studies suggest that homoacetogenesis cannot be enhanced until measures are taken to inhibit methanogens or increase hydrogen concentrations. In fact, the absence of methanogens is likely to increase the ruminal hydrogen concentrations, as evidenced by the increased hydrogen emissions from animals when methane inhibitors are applied (Mitsumori *et al.* 2012, Hristov *et al.* 2015). The research objective of this thesis was to explore the potential of resident homoacetogens in sheep rumen fluid to use hydrogen in the presence and absence of methanogens, employing methanogen-specific chemical inhibitors and without adding homoacetogens or excess hydrogen.

The first step towards studying homoacetogenesis was to find a specific-methanogen inhibitor. A number of known chemical inhibitors of methanogens were selected and screened against a variety of pure cultures of methanogens, homoacetogens and other bacteria, at a range of inhibitor concentrations. BES was found to inhibit only methanogens, but it has been reported in earlier studies that some rumen methanogens develop resistance to BES over a short period (Ungerfeld *et al.* 2004). When BES was introduced into sheep, methane reappeared after four days due to adaption of methanogens to BES (Immig *et al.* 1996). Therefore, BES

was considered to be a tool to investigate the short-term effects of methanogenesis inhibition on rumen fermentation pattern during *in vitro* studies, but not to be useful *in vivo*. Acetylene is known to inhibit methanogens specifically, and has been shown to inhibit methane production in batch experiments with rumen contents and in sheep (M. Tavendale, personal communication). Acetylene was selected as an inhibitor of methane formation *in vitro* (mixed culture) and *in vivo*. It would have been advantageous to find a specific homoacetogen inhibitor in order to study the significance of homoacetogenesis in the absence of methanogenesis, but no such inhibitor was found. In the absence of a specific inhibitor of homoacetogens, chloroform was selected, as it inhibits methanogenesis and partially inhibits homoacetogenesis.

In the past, the formation of acetate from CO_2 by incorporation of $^{14}CO_2$ or $^{13}CO_2$ into acetate has been used to measure homoacetogenesis (Prins & Lankhorst 1977, Morvan *et al.* 1994, Le Van *et al.* 1998). The principles of these methods were used to develop a protocol to estimate homoacetogenic activity in rumen contents by measuring the incorporation of $^{13}CO_2$ into ^{13}C-acetate in a short-term assay. The capacity of this activity to increase was estimated by adding BES to the assay, and chloroform was used to show that the measured activity was sensitive to inhibition, as would be expected for homoacetogenesis. However, measuring the appearance of label in acetate could be misleading. VFA inter-conversion results in label appearing in other VFA during infusion of labelled VFA (Leng & Brett 1966, Glinsky *et al.* 1976, Sharp *et al.* 1982, Bruce *et al.* 1987, Sutton *et al.* 2003, Ungerfeld & Kohn 2006, Nolan *et al.* 2014). Therefore, to confirm that the ^{13}C in the labelled acetate being measured was coming from direct CO_2 incorporation into acetate, and not due to inter-conversion from another VFA that had become labelled, short-term *in vitro* experiments were carried out using $^{13}C_2$-acetate, 2-$^{13}C_1$-propionate, $^{13}C_3$-propionate and $^{13}C_4$-butyrate. These experiments measured the fractional amounts of VFA inter-conversion between the three major VFA, and the data were used to correct for inter-conversion when calculating the amount of homoacetogenesis. $^{13}CO_2$ was rapidly incorporated into propionate, but inter-conversion of propionate to acetate could account for < 1% of the labelled acetate. This was also observed by others during infusion of labelled propionate, where the inter-conversion between propionate to acetate or vice-versa was relatively very small (Bergman *et al.* 1965, Leng & Brett 1966, Van Der Walt & Briel 1976).

Therefore, the short-term *in vitro* assay measured and confirmed the occurrence of homoacetogenesis in the sheep rumen, and showed that it could increase as an immediate response to an increased hydrogen concentration when methanogenesis was inhibited with BES. This homoacetogenesis was sensitive to chloroform addition *in vitro*, supporting the conclusion that this was true homoacetogenesis, as chloroform is known to inhibit homoacetogens (Conrad & Klose 2000, Scholten *et al.* 2000, Xu *et al.* 2010). Homobutyrogenesis, the formation of butyrate from CO_2 and H_2 by homoacetogens (Zeikus *et al.* 1980, Kerby *et al.* 1983, Hensley *et al.* 2012, Schiel-Bengelsdorf & Dürre 2012), was also reported in the rumen for the first time. Although it did not contribute greatly to total hydrogen use, it should be considered and explored in future experiments.

Homoacetogenesis was able to increase over a short-term incubation of rumen fluid with BES. It is possible that if methane could be inhibited for longer, homoacetogenesis might increase further. Studies have reported increases in acetate formation when homoacetogens or feed additives were added to *in vitro* systems (Chaucheyras *et al.* 1995, Nollet *et al.* 1997, Le Van *et al.* 1998, Nollet *et al.* 1998, Lopez *et al.* 1999, Yang *et al.* 2015). However, the contribution of acetate derived from homoacetogenesis was not clear. Therefore, in this study, the potential for rumen resident homoacetogens to increase in the absence of methanogens *in vitro* was explored without adding homoacetogens or feed additives, using a short-term assay that could distinguish formation of acetate derived from homoacetogenesis. An *in vitro* serial batch fermentation experiment was then carried out using sheep rumen fluid, to measure homoacetogenesis after 108 h of inhibition of methane production with acetylene, and study if homoacetogenesis increased further. For this, rumen contents were incubated in the presence and absence of acetylene, and serial transfers were carried out every 12 h to provide fresh substrate and buffer. Acetylene was added at each transfer to inhibit methanogenesis completely for 108 h. Homoacetogenesis was measured in the fresh rumen contents used to start the experiment (0 h), and after nine serial transfers in the presence and absence of acetylene (108 h). The dissolved hydrogen concentration and the estimated potential rate of homoacetogenic metabolism increased after nine serial transfers with inhibition of methane production in the presence of acetylene. Although homoacetogenesis did not increase further as compared to fresh rumen contents, the amount of activity was maintained, and therefore the homoacetogens must have

grown, since the experimental design meant that the original inoculum was diluted some 100,000-fold during the serial transfers. Homobutyrogenesis was measured in fresh rumen contents, but after nine serial transfers in the presence of acetylene, it was no longer detectable. Importantly, homoacetogenesis accounted for 2.32% of electron utilisation in serial transfers with acetylene as compared to 1.24% in non-inhibited serial transfers, and therefore was able to increase its contribution as an electron sink in the absence of methanogens. Propionate was the major electron sink in the absence of methanogenesis, and increased from utilising approximately 42% of total electrons in fresh rumen contents (pre-treatment) to utilising 58% of total electrons after nine serial transfers with no inhibitor and 88% of total electrons after nine serial transfers in the presence of acetylene. Increases in electron flow towards propionate have also been observed during inhibition of methanogenesis in batch and continuous culture experiments (Chalupa *et al.* 1980, van Nevel & Demeyer 1981, Watanabe *et al.* 2010, O'Brien *et al.* 2014, Ungerfeld 2015). This suggests that propionate is one of the major electron sink in the absence of methanogens.

In order to provide a more favourable environment for homoacetogens to grow and increase in the rumen, a trial was carried out in which methane production from sheep was inhibited for five days by daily dosing with an acetylene-generating bolus. Other group of sheep was administered a chloroform-cyclodextrin paste in a capsule-form to inhibit both methanogens and homoacetogens, and control sheep were used as an experimental covariant. The VFA profile in the sheep rumens, and the associated archaeal and bacterial communities changed following inhibition of methane production. Formyltetrahydrofolate synthetase gene sequences in sheep rumen samples were identified using a homoacetogen FTHFS profile hidden Markov model (HoF-HMM). No increase in FTHFS gene sequences with high or intermediate HMMER bit score was observed during five days of inhibition of methane production in sheep using acetylene. Homoacetogenesis was measured prior to treatment (day 1) and during the treatment period (day 8). As compared to day 1 and control sheep, there was increase in homoacetogenesis in the rumens of acetylene-treated sheep. Homobutyrogenesis did not increase significantly during inhibition of methane production in acetylene-treated sheep. In chloroform-treated sheep there was also an increase in homoacetogenesis, which might be due to the low concentration of chloroform administered to the sheep or resistance of some homoacetogens towards chloroform. Even so, it was less than in the acetylene-

treated sheep, as expected. The increase in homoacetogenesis with acetylene also resulted in an increase in electron utilisation by homoacetogenesis to 6.53% as compared to day 1 which was 1.47% (pre-treatment) in control sheep. Homoacetogenesis and homobutyrogenesis together accounted for 7.08% of electron use in the acetylene-treated sheep, and only 1.97% in the control sheep or in sheep prior to treatment with the inhibitor. It appears unlikely that homoacetogenesis will be a major electron sink when methane formation is inhibited, although it was more important than net hydrogen gas production in the study reported here. Based on the results from the studies presented here, it seems that the hypothesis (Boccazzi & Patterson 1996, Joblin 1999, Ungerfeld & Kohn 2006, Gagen *et al.* 2015, Ungerfeld 2015) that homoacetogens could fulfil the role of hydrogen users that methanogens occupied, after the latter group of microbes is inhibited, might be true. However, it did not become a major electron or hydrogen sink. Instead, as observed in the serial batch transfer *in vitro* experiment and in the animal trial, propionate became the major electron sink, and there was a dramatic increase in the estimated electron flow to propionate from approximately 35% without inhibitor to 58% in the absence of methanogenesis in rumen. The significance of propionate as an electron sink has been discussed in detail by Ungerfeld in the methanogenesis-inhibited rumen fermentation, where electron flow to propionate increased from 26.7% to 61.7% (O'Brien *et al.* 2014, Ungerfeld 2015).

Propionate is the second most important electron sink after methane and it competes with methanogens to use electrons (Czerkawski 1986, Wolin *et al.* 1997). However, in the absence of methanogens it becomes the major electron sink as observed in the present study and as discussed above. Moreover, it is known that when methanogens are inhibited, the increase in hydrogen concentration thermodynamically favours propionate production (Janssen 2010). It was also observed, during inhibition of methane formation both *in vitro* and *in vivo*, that there was always an increase in the proportion of propionate as compared to other products. Propionate formation from feed fermentation is generally not a direct hydrogen-utilising pathway; instead it is an alternative to hydrogen formation as it uses reducing equivalents or electrons formed, thereby leading to less hydrogen formation and less methane (Janssen 2010). However, propionate formation through the randomizing pathway can also incorporates dihydrogen in the reduction

of fumarate to succinate in some organisms (Henderson 1980, Asanuma *et al*. 1998). Re-direction of electrons or hydrogen to propionate would be beneficial for the animal, as it favours milk protein production, as observed during ruminal infusion of propionate in cows (Sheperd & Combs 1998). Therefore, it will be beneficial to explore propionate formation further as a significant electron sink in the absence of methanogenesis.

Effects of methane inhibition on animal production have been explored, but this was not been studied in the present *in vivo* trial, as this trial was mainly designed to measure homoacetogenesis in the presence and absence of methanogenesis during five days of inhibition of methane production. Inhibiting methane production by 94-95% in cows using chloroform resulted in no effects on live weights of the animals (Knight *et al.* 2011). Inhibition of methane production by more than 80% in goats using bromochloromethane (BCM) had no effects on dry matter intake (DMI) and feed digestibility. However, it increased the ruminal hydrogen concentration (Mitsumori *et al.* 2012). The inhibited rumen seemed to adapt to this increased ruminal hydrogen concentration by shifting fermentation to more propionate formation and there was a 20% increase in the flow of metabolic hydrogen into VFA (Mitsumori *et al.* 2012). In another experiment, treatment with BCM reduced methane production by 33% in goats, and resulted in a 36% increase in milk yield, probably due to the shift in rumen fermentation towards more propionate and an increase in VFA production. The increase in milk yield was not accompanied by statistically significant changes in its composition (Abecia *et al.* 2012). Inhibition of methane production in cows using a methane production inhibitor, 3-nitrooxypropanol (3-NOP), resulted in a 30% reduction in methane production over 12-week period, but without affecting feed intake or milk production and composition (Hristov *et al.* 2015). 3-NOP was found to have no effects on feed digestabilty and milk production in previous studies as well (Haisan *et al*. 2014, Romero-Perez *et al*. 2014, Martinez-Fernandez *et al*. 2014). Milk fat content and yield were not affected by 3-NOP, but it increased the milk lactose and protein contents by about 3%. The energy spared from methane appeared to be partially used for tissue synthesis and this lead to 80% greater increases in body weight gains compared to controls, which is beneficial for animal's energy balance and overall animal performance. Inhibition of methane production lead to 64-fold increase in hydrogen emission. However, the amount of hydrogen emitted was still

only a fraction of the amount available from inhibtion of methane production (Hristov *et al.* 2015). This further suggested adaptation of rumen and reduced hydrogen formation or redirection of hydrogen to alternative hydrogen sinks.

Incorporation of hydrogen/electrons into alternative hydrogen/electron sinks, i.e., homoacetogenesis or additional propionate formation therefore can lead to energetic and nutritional consequences for the animals during inhibition of methane production and could have additional metabolic consequences for the ruminants including post-absorptive metabolism arising due to changes in VFA profile (DiCostanzo *et al*. 1999, Ungerfeld 2013). Increases in hydrogen/electrons utilisation by homoacetogenesis could favour milk production, but increase the risk of ketosis in forage-based and fat-supplemented diets, and decrease in rumen pH which could also affect DMI (Ungerfeld 2013). Greater propionate formation could increase milk protein production and decrease risk of ketosis in animals fed ketogenic diets, but less beneficial for animals with metabolically constrained feed intake such as feedlot steers fed high concentrate diets, which could result in decreased DMI (Ungerfeld 2013). However, effects of redirecting hydrogen/electrons from methane to homoacetogenesis or propionate production over long-term methane inhibition on animal production, physiology and energy balance still needs to be explored and compared. Physico-chemical control of hydrogen/electron utilisation by homoacetogenesis or increased propionate production dependent on enzymes or substrate thermodynamics could help design methodology to achieve successful and balanced utilisation of hydrogen/electrons into these two pathways (Ungerfeld 2013).

7.2 Recommendations for future work

The research presented here showed that there was an increase in homoacetogenesis during inhibition of methane production in sheep. A number of further lines of investigation suggest themselves, based on the studies presented in this thesis.

- No significant increase in homoacetogenesis was observed during the *in vitro* serial batch fermentation experiment treated with a methanogenesis inhibitor. This is likely to be a result of unfavourable conditions in the culture system. But homoacetogens were able to maintain their population over nine serial transfers and there was an increase in homoacetogenesis in the presence of acetylene-

treated as compared to non-inhibited serial transfers. It has been observed that addition of homoacetogens in the presence of a methane inhibitor to the *in vitro* system enhance acetate formation from H_2 and CO_2 (Le Van *et al.* 1998). Therefore, addition of homoacetogens to the *in vitro* serial batch fermentation system along with methanogenesis inhibitor could be used to see if homoacetogenesis increases, as this will provide increased numbers of homoacetogens *in vitro*.

- Due to the unavailability of a long-term methanogenesis inhibitor, a long-term *in vivo* trial could not be carried out to determine if homoacetogenesis increased further, or remained constant or decreased over further inhibition of methane production. The availability of 3-nitrooxypropanol (Hristov *et al.* 2015), a long-term inhibitor of methanogenesis, could be used to test the significance of homoacetogenesis as an alternative hydrogen and electron sink in animals over a longer period. The tools and methods developed in this thesis can be used to explore the changes in rumen fermentation and homoacetogenesis.
- If methane could be inhibited for a longer-term using a methanogenesis-specific inhibitor, such as 3-nitrooxypropanol (Hristov *et al.* 2015), homoacetogens could also be inoculated (Fonty *et al.* 2007) into the rumen. Homoacetogenesis could then be measured to test if added homoacetogens could increase homoacetogenesis, and use hydrogen available in the absence of methanogens, and take over hydrogen-utilisation over time.
- In the present thesis, methods were developed to measure homoacetogenesis employing $NaH^{13}CO_3$ in short-term *in vitro* incubation. In future, $NaH^{13}CO_3$ could be administered into the animals in the presence and absence of methanogenesis inhibitors, to measure homoacetogenesis in animals directly.
- Formyltetrahydrofolate synthetase (FTHFS) and acetyl-CoA synthase (ACS) gene sequences in bovine rumen and tammar wallaby gut contents clustered between the *Lachnospiraceae* and *Clostridiaceae* acetogens but were not close to sequences from cultured isolates (Gagen *et al.* 2010). During rumen fermentation involving the addition of the exogenous acetogen strain TWA4 and *Saccharomyces cerevisiae* fermentation product XP, acetyl-CoA synthase (ACS) genes were sequenced to investigate acetogen diversity. *Lachnospiraceae*-affiliated ACS genes was found to be predominant in all communities, but were not highly similar to amino acid sequences of ACS genes from cultured isolates. More *Eubacterium limosum*-like sequences, more

Acetitomaculum ruminis-like sequences and less *Eubacterium limosum*-like sequences were identified in XP, TWA4 and TWA4 plus XP treatments respectively (Yang *et al.* 2015). The rumen samples from methanogen inhibitor treated and untreated sheep could be used for the detailed analysis and phylogeny of formyltetrahydrofolate synthetase (FTHFS) and acetyl-CoA synthase (ACS) gene sequences having high similarity scores to the sequences from known homoacetogens (Gagen *et al.* 2010, Henderson *et al.* 2010, Yang *et al.* 2015), to identify the homoacetogens in the rumen of methane-inhibited and uninhibited sheep.

- Metatranscriptomic analyses of rumen microbial communities in the presence and absence of methanogen inhibitors could be used to show that homoacetogens express genes like FTHFS and ACS, helping to confirm their activity. This would also help identify which potential homoacetogens detected in the rumen (e.g., Gagen *et al*. 2010, Henderson *et al*. 2010, Yang *et al*., 2015) were actually active.
- RNA stable isotope probing (RNA-SIP) has been used to identify bacteria associated with metabolism of carbon dioxide and hydrogen in the kangaroo foregut. The reductive acetogen *Blautia coccoides* and members of the genera *Prevotella*, *Oscillibacter* and *Streptococcus*, not known to be homoacetogens, were reportedly involved in the metabolism of carbon dioxide and hydrogen in the kangaroo foregut (Godwin *et al.* 2014). Therefore, RNA stable isotope probing (RNA-SIP) could be used to confirm the identity of active homoacetogens in the methane inhibited and uninhibited sheep rumen.
- In the absence of homoacetogens being the significant alternative hydrogen utilisers, the role of propionate as an alternative electron sink must be explored, as it represents the most important electron sink in the absence of methanogens. Factors like the change in microbial biomass (protein) synthesis and the impacts of this on animal production should be investigated, especially on forage-based diets.

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Appendix 1

The correction factors in the calculation of fractional amounts of VFA inter-conversion (Chapter 4) were derived from the isobaric ratios of the species containing one or more less labels relative to an unlabelled ion with a natural abundance binomial distribution (Table A.1).

Table A.1 Mole percent excess (MPE_i) calculations for ^{13}C-labelled isomers.

Ion peak area ratio	MPE calculations
$^{13}C_2$-acetate isomers	
$R_{1e} = (R_1 - R_{10})$	$MPE_0 = 1/(R_{1e} + R_{2e} + 1) \times 100$
$R_{2e} = (R_2 - R_{20} - ¾ \times R_{1e} \times R_{10})$	$MPE_1 = R_{1e}/(R_{1e} + R_{2e} + 1) \times 100$
	$MPE_2 = R_{2e}/(R_{1e} + R_{2e} + 1) \times 100$
$^{13}C_3$-propionate isomers	
$R_{1e} = (R_1 - R_{10})$	$MPE_0 = 1/(R_{1e} + R_{2e} + R_{3e} + 1) \times 100$
$R_{2e} = R_2 - R_{20} - 4/5 \times R_{1e} \times R_{10})$	$MPE_1 = R_{1e}/(R_{1e} + R_{2e} + R_{3e} + 1) \times 100$
$R_{3e} = (R_3 - R_{30} - 3/5 \times R_{2e} \times R_{10} - 3/5 \times R_{1e} \times R_{20})$	$MPE_2 = R_{2e}/(R_{1e} + R_{2e} + R_{3e} + 1) \times 100$
	$MPE_3 = R_{3e}/(R_{1e} + R_{2e} + R_{3e} + 1) \times 100$
$^{13}C_4$-butyrate isomers	
$R_{1e} = (R_1 - R_{10})$	$MPE_0 = 1/(R_{1e} + R_{2e} + R_{3e} + R_{4e} + 1) \times 100$
$R_{2e} = (R_2 - R_{20} - 5/6 \times R_{1e} \times R_{10})$	$MPE_1 = R_{1e}/(R_{1e} + R_{2e} + R_{3e} + R_{4e} + 1) \times 100$
$R_{3e} = (R_3 - R_{30} - 4/6 \times R_{2e} \times R_{10} - 4/6 \times R_{1e} \times R_{20})$	$MPE_2 = R_{2e}/(R_{1e} + R_{2e} + R_{3e} + R_{4e} + 1) \times 100$
$R_{4e} = (R_4 - R_{40} - 3/6 \times R_{3e} \times R_{10} - 12/30 \times R_{2e} \times R_{20} - 3/6 \times R_{1e} \times R_{30})$	$MPE_3 = R_{3e}/(R_{1e} + R_{2e} + R_{3e} + R_{4e} + 1) \times 100$
	$MPE_4 = R_{4e}/(R_{1e} + R_{2e} + R_{3e} + R_{4e} + 1) \times 100$

For ion ratios R, the first subscripted number refers to the number of ^{13}C carbons contained by the ion species with the highest mass. The second subscripted number "0" infers a control or background sample ion ratio. The subscripted letter "e" refers to the ion ratio in excess to the ratio contributions from species

containing labelled carbons substituted with naturally occurring ^{13}C carbons which includes the control or background sample ion ratios. All ion ratios were calculated from the ion's peak area relative to the universally ^{12}C substituted ion peak area.

For example for acetate R_1 and R_{10} correspond to ion peak area ratios for ions m/z 108:107 for sample and control respectively; R_2 and R_{20} for ions m/z 109:107 for sample and control respectively; R_3 and R_{30} for ions m/z 110:107 for sample and control respectively; R_4 and R_{40} for ions m/z 111:107 for sample and control respectively. MPE_i is mole percent excess for any species containing i ^{13}C labelled carbons is as a percent of the total labelled and unlabelled species present.

Appendix 2

Table A.2.1 Mean amounts and proportions of products produced in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation (mixed culture) system in the absence of inhibitor, and presence of acetylene over time.

	No inhibitor									Acetylene								
	Time (h)									Time (h)								
Products in mmol/bottle	**12**	**24**	**36**	**48**	**60**	**72**	**86**	**96**	**108**	**12**	**24**	**36**	**48**	**60**	**72**	**86**	**96**	**108**
Methane	0.259	0.194	0.171	0.138	0.123	0.124	0.118	0.135	0.132	0.018	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Hydrogen	0.006	0.008	0.013	0.015	0.017	0.020	0.024	0.022	0.024	0.127	0.095	0.068	0.052	0.042	0.034	0.033	0.042	0.058
Total gas	2.657	2.238	2.269	2.163	2.221	2.213	2.218	2.330	2.280	2.336	2.159	2.168	2.056	2.006	1.967	1.964	2.116	2.107
Acetate	1.765	1.490	1.244	1.145	1.266	1.065	1.046	1.132	1.105	1.357	1.117	0.938	0.771	0.938	0.863	0.865	0.901	0.870
Propionate	0.745	0.788	0.831	0.946	1.107	1.005	0.961	0.967	0.938	0.937	1.134	1.231	1.206	1.434	1.432	1.396	1.397	1.291
Butyrate	0.337	0.237	0.181	0.163	0.204	0.179	0.181	0.175	0.153	0.394	0.283	0.217	0.157	0.157	0.127	0.097	0.073	0.049
Isobutyrate	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.021	0.001	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Isovalerate	0.033	0.007	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.027	0.001	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Valerate	0.076	0.080	0.080	0.085	0.105	0.110	0.115	0.125	0.127	0.078	0.081	0.083	0.081	0.092	0.089	0.083	0.081	0.074
Caproate	< 0.01	< 0.01	< 0.01	< 0.01	0.014	0.025	0.032	0.034	0.034	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Lactate	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Formate	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.142	0.111	0.087	0.033	0.038	0.091	0.103	0.101	0.119
Ethanol	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.047	< 0.01	< 0.01	< 0.01	< 0.01	0.044	0.051	0.070	0.102
Total VFA	2.984	2.611	2.331	2.334	2.684	2.372	2.325	2.422	2.350	2.812	2.616	2.454	2.196	2.606	2.496	2.424	2.36	2.269
Proportion																		
Acetate	0.591	0.570	0.534	0.490	0.473	0.449	0.450	0.467	0.470	0.482	0.427	0.381	0.349	0.360	0.346	0.356	0.369	0.381
Propionate	0.250	0.302	0.356	0.406	0.413	0.425	0.414	0.400	0.400	0.333	0.434	0.503	0.551	0.551	0.574	0.576	0.574	0.572
Butyrate	0.113	0.091	0.078	0.070	0.074	0.075	0.077	0.072	0.065	0.140	0.108	0.088	0.072	0.060	0.051	0.040	0.030	0.021
Acetate: propionate	2.369	1.889	1.498	1.209	1.147	1.059	1.086	1.170	1.177	1.448	0.984	0.760	0.635	0.653	0.603	0.619	0.644	0.668
SEM[a] (n = 8[b])																		
Methane	0.004	0.002	0.003	0.002	0.002	0.001	0.002	0.002	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

	No inhibitor									Acetylene								
	Time (h)									Time (h)								
Products in mmol/bottle	**12**	**24**	**36**	**48**	**60**	**72**	**86**	**96**	**108**	**12**	**24**	**36**	**48**	**60**	**72**	**86**	**96**	**108**
Hydrogen	<0.001	<0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.004	0.003	0.003	0.002	0.002	0.002	0.002	0.002	0.003
Total gas	0.013	0.014	0.016	0.036	0.019	0.021	0.019	0.017	0.016	0.028	0.017	0.019	0.013	0.014	0.013	0.011	0.020	0.011
Acetate	0.031	0.051	0.013	0.023	0.064	0.045	0.041	0.031	0.038	0.029	0.032	0.035	0.043	0.023	0.018	0.024	0.025	0.059
Propionate	0.014	0.020	0.008	0.014	0.068	0.039	0.026	0.016	0.024	0.015	0.020	0.024	0.032	0.020	0.015	0.019	0.022	0.048
Butyrate	0.005	0.007	0.003	0.004	0.029	0.018	0.010	0.006	0.008	0.011	0.011	0.009	0.007	0.005	0.002	0.004	0.004	0.006
Isobutyrate	-	-	-	-	-	-	-	-	-	0.001	0.001	-	-	-	-	-	-	-
Isovalerate	<0.001	<0.001	-	-	-	-	-	-	-	0.001	0.001	-	-	-	-	-	-	-
Valerate	0.001	0.002	<0.001	0.001	0.006	0.007	0.006	0.005	0.006	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.003
Caproate	-	-	-	-	<0.001	<0.001	0.001	0.001	0.001	-	-	-	-	-	-	-	-	-
Lactate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Formate	-	-	-	-	-	-	-	-	-	0.005	0.005	0.005	0.005	0.002	0.003	0.007	0.002	0.003
Ethanol	-	-	-	-	-	-	-	-	-	0.016	-	-	-	-	0.001	0.002	0.004	0.006
Total VFA	0.050	0.078	0.020	0.040	0.166	0.108	0.081	0.052	0.070	0.058	0.066	0.071	0.083	0.046	0.034	0.045	0.050	0.116
Proportion																		
Acetate	0.001	0.004	0.001	0.002	0.005	0.003	0.004	0.003	0.003	0.002	0.002	0.004	0.007	0.003	0.003	0.003	0.004	0.007
Propionate	0.001	0.003	0.002	0.002	0.002	0.003	0.004	0.005	0.005	0.002	0.004	0.005	0.007	0.002	0.002	0.003	0.003	0.009
Butyrate	0.001	0.001	0.001	0.001	0.005	0.004	0.002	0.002	0.003	0.001	0.002	0.001	0.001	0.002	0.001	0.001	0.001	0.002
Acetate: propionate	0.015	0.028	0.012	0.009	0.017	0.008	0.019	0.019	0.020	0.013	0.012	0.015	0.020	0.008	0.007	0.008	0.010	0.023
p-value compared to no inhibitor at same time (h)																		
Methane										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Hydrogen										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0007	0.0001	0.0001
Total gas										0.0001	0.0125	0.0005	0.0002	0.0001	0.0001	0.0001	0.0001	0.0001
Acetate										0.0001	0.0001	0.0001	0.0001	0.0001	0.0011	0.0001	0.0002	0.0001
Propionate										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Butyrate										0.1032	0.0983	0.1645	1	0.0331	0.0009	0.0001	0.0001	0.0001
Isobutyrate										0.0001	0.0001	0.0001	0.0001	1	1	1	1	0.0001
Isovalerate										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Valerate										1	1	1	1	0.0553	0.0002	0.0001	0.0001	0.0001
Caproate										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

	No inhibitor									Acetylene								
	Time (h)									Time (h)								
Products in mmol/bottle	**12**	**24**	**36**	**48**	**60**	**72**	**86**	**96**	**108**	**12**	**24**	**36**	**48**	**60**	**72**	**86**	**96**	**108**
Lactate										1	0.8536	0.0001	0.1255	0.4762	0.1543	1	0.0221	0.0267
Formate										0.0001	0.0001	1	1	1	1	1	1	1
Ethanol										0.0849	1	0.009	1	1	1	0.0051	0.025	0.0044
Total VFA										1	1	1	1	1	1	1	1	1
Proportion																		
Acetate										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Propionate										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Butyrate										0.0001	0.0001	0.0038	1	0.0001	0.0001	0.0001	0.0001	0.0001
Acetate: propionate										0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

[a]Standard error of the mean.

[b]No. of experimental replicates.

Table A.2.2 The number of reads and OTUs for each sample sequence for archaea and bacteria in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor, and presence of acetylene over time.

		Archaea		Bacteria	
Replicate	**Time (h)**	**No. of reads**	**No. of OTUs**	**No. of reads**	**No. of OTUs**
No inhibitor					
1	12	640	42	9436	4977
2	12	2498	74	6091	3198
3	12	1948	66	9381	3497
4	12	1788	76	2901	1687
5	12	963	48	8059	2662
6	12	3181	81	4532	2651
7	12	1971	66	3068	1461
8	12	539	34	6952	3608
1	36	1191	54	12005	5015
2	36	1720	74	6935	3065
3	36	1988	95	7910	3218
4	36	3231	105	4526	2273
5	36	1602	77	5147	2002
6	36	1547	68	3807	1450
7	36	1130	73	8460	3601
8	36	699	49	9800	2804
1	60	2136	111	15242	2984
2	60	932	71	5700	1481
3	60	1155	69	4507	1185
4	60	2123	107	4379	1515
5	60	1820	128	5596	1954
6	60	1192	94	8619	2908
7	60	535	62	6147	2029
8	60	1408	106	8697	2262
1	84	1549	68	21611	3493
2	84	1708	71	7195	2214
3	84	2667	107	4759	1253
4	84	1324	80	6034	1543
5	84	2323	91	13024	3722
6	84	564	58	7081	2116
7	84	1805	104	10876	3203
8	84	1346	86	7894	2055
1	108	1211	65	6768	2542
2	108	1125	63	13462	4450
3	108	1057	69	7843	2994
4	108	2477	106	9342	3419
5	108	1114	65	5336	2020
6	108	1499	58	5016	2016
7	108	761	54	8987	2575
8	108	948	53	15892	2906

		Archaea		Bacteria	
Replicate	**Time (h)**	**No. of reads**	**No. of OTUs**	**No. of reads**	**No. of OTUs**
Acetylene					
1	12	783	40	6262	3100
2	12	1465	55	9190	4882
3	12	2382	60	11872	5476
4	12	640	27	4132	2105
5	12	550	33	6034	2698
6	12	1266	52	6460	2508
7	12	752	30	5649	3080
8	12	469	26	7481	3657
1	36	2067	75	6585	2973
2	36	880	41	5213	2492
3	36	1158	49	5972	2670
4	36	920	42	9927	2641
5	36	1691	60	5054	2305
6	36	839	44	4151	1953
7	36	436	33	6657	2147
8	36	758	49	5233	2087
1	60	2221	49	5445	1552
2	60	1350	52	7604	2272
3	60	1404	43	5743	1833
4	60	1889	53	6437	1598
5	60	2320	62	3833	1256
6	60	1069	46	8493	2427
7	60	1573	56	8246	2410
8	60	647	48	7125	2447
1	84	1148	52	5913	1672
2	84	759	37	6686	1104
3	84	1416	53	5228	1111
4	84	1482	47	8808	2207
5	84	1018	47	7942	1651
6	84	403	32	7481	1650
7	84	773	43	10721	2814
8	84	527	39	8724	2256
1	108	742	37	12511	2281
2	108	378	29	7165	1788
3	108	423	31	6034	1068
4	108	368	26	6601	1218
5	108	437	37	7994	1871
6	108	369	35	8943	1693
7	108	271	36	9635	2496
8	108	949	38	3484	833

Table A2.3 The most abundant and prevalent rumen bacterial genus-level groups in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the absence of inhibitor during *in vitro* serial transfer over time. The 19 most abundant groups (mean relative abundance > 1% in any one sample) are indicated in bold font.

	Mean relative abundance (%)					**SEM[a] (*n* = 8[b])**				
	Time (h)					**Time (h)**				
Taxa	**12**	**36**	**60**	**84**	**108**	**12**	**36**	**60**	**84**	**108**
Unclassified[c] *Coriobacteriaceae*	0.401	0.179	0.131	0.135	0.226	0.086	0.047	0.023	0.028	0.021
Unclassified *Bacteroidales*	5.289	3.801	4.366	4.791	6.737	0.260	0.094	0.383	0.394	0.324
BS11 (*Bacteroidales*)	0.693	0.263	0.218	0.337	0.623	0.093	0.030	0.030	0.055	0.074
BF311 (*Bacteroidaceae*)	0.244	0.278	0.501	0.692	0.748	0.043	0.031	0.054	0.066	0.063
Paludibacter	0.013	0.025	0.211	0.129	0.038	0.008	0.007	0.020	0.023	0.013
Unclassified *Prevotellaceae*	0.509	0.489	0.422	0.297	0.401	0.076	0.048	0.053	0.029	0.032
Prevotella	42.96	35.00	21.49	20.45	25.26	1.947	1.389	0.837	1.442	0.810
RF16 (*Bacteroidales*)	0.997	0.330	0.410	0.385	0.317	0.094	0.058	0.053	0.039	0.029
S24-7 (*Bacteroidales*)	2.240	0.767	0.270	0.325	0.823	0.180	0.071	0.036	0.038	0.058
Unclassified [*Paraprevotellaceae*][d]	0.866	0.563	0.574	0.539	0.818	0.119	0.050	0.063	0.079	0.092
CF231 [*Paraprevotellaceae*]	0.740	0.402	0.307	0.343	0.442	0.058	0.035	0.037	0.036	0.049
YRC22 [*Paraprevotellaceae*]	1.505	1.232	1.066	0.592	0.776	0.116	0.072	0.087	0.064	0.055
YS2 (*Cyanobacteria*)	0.233	0.216	0.299	0.087	0.058	0.033	0.025	0.029	0.014	0.015
Fibrobacter	1.275	2.219	2.710	2.160	1.129	0.118	0.149	0.256	0.161	0.105
Streptococcus	0.141	6.076	34.67	35.92	11.56	0.027	0.996	2.244	4.635	2.038
Unclassified *Clostridiales*	7.235	4.957	3.645	3.357	4.211	0.618	0.262	0.275	0.195	0.243
Unclassified *Christensenellaceae*	0.440	0.108	0.067	0.088	0.083	0.044	0.022	0.015	0.016	0.015
Clostridium	0.568	0.530	0.337	0.278	0.371	0.051	0.045	0.040	0.037	0.033
Unclassified *Lachnospiraceae*	5.116	6.518	4.203	5.523	8.638	0.251	0.403	0.163	0.447	0.346
Butyrivibrio	1.481	2.248	1.295	1.406	2.582	0.204	0.158	0.106	0.122	0.074
Coprococcus	1.352	2.281	1.095	1.147	1.414	0.098	0.076	0.103	0.099	0.084
Pseudobutyrivibrio	0.585	4.037	3.302	2.842	5.062	0.030	0.300	0.107	0.295	0.325
Shuttleworthia	0.152	0.672	0.573	0.376	0.311	0.019	0.052	0.065	0.055	0.049
Unclassified *Ruminococcaceae*	7.570	3.308	2.297	2.680	4.249	0.532	0.199	0.234	0.259	0.270
Oscillospira	0.268	0.198	0.189	0.140	0.215	0.038	0.037	0.036	0.028	0.038
Ruminococcus	2.198	2.390	1.561	1.467	1.685	0.083	0.105	0.179	0.202	0.157
Unclassified *Veillonellaceae*	3.964	1.916	0.660	0.566	0.692	0.147	0.097	0.080	0.071	0.045
Anaerovibrio	0.136	0.738	1.123	1.309	1.672	0.027	0.075	0.121	0.106	0.091

	Mean relative abundance (%)					SEM[a] (n = 8[b])				
	Time (h)					Time (h)				
Taxa	**12**	**36**	**60**	**84**	**108**	**12**	**36**	**60**	**84**	**108**
Selenomonas	1.655	3.568	1.792	1.575	2.721	0.103	0.219	0.132	0.161	0.265
Succiniclasticum	1.285	2.218	1.479	1.552	2.306	0.105	0.155	0.075	0.154	0.110
Unclassified [*Mogibacteriaceae*]	0.719	0.279	0.231	0.216	0.373	0.085	0.026	0.036	0.028	0.059
Mogibacterium	0.554	0.345	0.247	0.223	0.411	0.077	0.040	0.030	0.031	0.037
Bulleidia	0.978	0.802	0.493	0.611	1.715	0.102	0.069	0.018	0.052	0.121
RFN20 *Erysipelotrichaceae*	0.352	0.376	0.425	0.395	0.534	0.058	0.030	0.050	0.059	0.042
Unclassified *Alphaproteobacteria*	0.118	0.366	0.391	0.857	1.832	0.022	0.054	0.040	0.111	0.151
Unclassified *Rickettsiales*	0.223	0.142	0.127	0.124	0.211	0.059	0.014	0.025	0.013	0.019
Unclassified *Succinivibrionaceae*	0.029	0.389	0.103	0.050	0.088	0.005	0.070	0.013	0.012	0.021
SR1 (Bacteria)	0.398	0.151	0.033	0.017	0.028	0.041	0.022	0.011	0.008	0.007
Sphaerochaeta	0.023	0.102	0.198	0.412	0.445	0.008	0.014	0.030	0.088	0.036
Treponema	0.514	5.717	3.148	2.921	4.523	0.040	0.363	0.352	0.292	0.205
F16 (TM7)	0.547	0.378	0.141	0.205	0.556	0.064	0.062	0.023	0.033	0.084
Unclassified *Anaeroplasmataceae*	0.039	0.210	0.218	0.377	0.382	0.010	0.027	0.039	0.061	0.090
Anaeroplasma	0.597	0.653	0.482	0.232	0.383	0.057	0.025	0.059	0.013	0.042
RF39 (*Mollicutes*)	1.010	1.481	1.397	0.863	0.663	0.055	0.144	0.142	0.084	0.050
Others[e]	1.791	1.084	1.103	1.006	1.682	0.104	0.048	0.072	0.084	0.136

[a]Standard error of the mean.

[b]No. of experimental replicates.

[c]Indicates the group contains sequences not classified down to the genus level.

[d]Square brackets indicate suggested but not verified taxon designations;

[e]A small proportion of sequences that were not present at > 0.5% in even one of the samples.

Table A2.4 The most abundant and prevalent rumen bacterial genus-level groups in sheep rumen contents incubated in the serially-transferred *in vitro* batch fermentation system in the presence of acetylene over time. The 19 most abundant groups (mean relative abundance > 1% in any one sample) are indicated in bold font and the sequences which have significant p-value < 0.05 are also indicated in bold font.

	Mean relative abundance (%)					**SEM[a] (*n* = 8[b])**					**p-value (compared to no inhibitor at same time (h))**				
	Time (h)					**Time (h)**					**Time (h)**				
Taxa	**12**	**36**	**60**	**84**	**108**	**12**	**36**	**60**	**84**	**108**	**12**	**36**	**60**	**84**	**108**
Unclassified[c] *Coriobacteriaceae*	0.164	0.197	0.164	0.089	0.112	0.022	0.035	0.029	0.025	0.026	**0.0009**	1	1	0.9299	0.0515
Unclassified *Bacteroidales*	4.970	6.119	7.377	6.019	6.172	0.231	0.296	0.311	0.680	0.299	1	**0.0001**	**0.0001**	0.1363	1
BS11 (*Bacteroidales*)	0.215	0.036	0.010	0.013	0.017	0.043	0.014	0.007	0.004	0.007	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
BF311 (*Bacteroidaceae*)	0.442	0.719	0.578	0.374	0.190	0.050	0.074	0.038	0.069	0.044	**0.0157**	**0.0001**	1	**0.0022**	**0.0001**
Paludibacter	0.010	0.278	1.543	1.414	0.711	0.004	0.035	0.122	0.245	0.085	1	**0.0001**	**0.0001**	**0.0001**	**0.0001**
Unclassified *Prevotellaceae*	0.473	0.352	0.381	0.421	0.475	0.038	0.041	0.048	0.069	0.043	1	0.2911	1	0.6914	1
Prevotella	48.66	31.67	21.55	18.66	18.47	1.060	0.734	0.663	1.783	0.707	0.1302	0.6368	1	0.8606	**0.0003**
RF16 (*Bacteroidales*)	0.995	0.292	0.273	0.104	0.075	0.058	0.030	0.032	0.027	0.020	1	1	0.1009	**0.0001**	**0.0001**
S24-7 (*Bacteroidales*)	2.089	1.400	0.823	0.594	0.534	0.156	0.054	0.131	0.110	0.066	1	**0.0003**	**0.0001**	**0.0488**	0.0688
Unclassified [*Paraprevotellaceae*][d]	0.780	1.055	1.339	0.776	0.536	0.044	0.084	0.091	0.129	0.022	1	**0.0007**	**0.0001**	0.3256	0.1309
CF231 [*Paraprevotellaceae*]	0.745	0.473	0.340	0.208	0.226	0.057	0.045	0.029	0.032	0.033	1	1	1	**0.0149**	**0.0002**
YRC22 [*Paraprevotellaceae*]	1.537	1.784	1.132	1.031	1.797	0.148	0.091	0.077	0.124	0.132	1	**0.0065**	1	**0.0011**	**0.0001**
YS2 (*Cyanobacteria*)	0.106	0.039	0.190	0.139	0.024	0.011	0.010	0.045	0.036	0.005	**0.0411**	**0.0001**	**0.0276**	0.9609	0.763
Fibrobacter	1.442	1.467	7.137	5.067	0.453	0.179	0.078	0.368	1.524	0.050	1	0.1703	**0.0001**	**0.0001**	**0.003**
Streptococcus	0.076	9.295	28.85	45.26	49.66	0.019	1.201	1.974	3.916	1.492	1	0.13	0.6686	0.1272	**0.0001**
Unclassified *Clostridiales*	5.833	4.842	2.777	1.814	1.568	0.306	0.282	0.158	0.230	0.072	0.0696	1	0.0548	**0.0001**	**0.0001**
Unclassified *Christensenellaceae*	0.201	0.053	0.057	0.058	0.045	0.031	0.010	0.016	0.014	0.011	**0.0001**	0.5425	1	0.301	0.7807
Clostridium	0.445	0.254	0.076	0.040	0.075	0.045	0.027	0.017	0.007	0.026	0.7952	**0.0004**	**0.0001**	**0.0001**	**0.0001**
Unclassified *Lachnospiraceae*	4.283	4.342	2.704	1.947	2.129	0.356	0.186	0.131	0.347	0.159	0.1739	**0.0001**	**0.0001**	**0.0001**	**0.0001**
Butyrivibrio	1.525	2.721	2.217	1.406	1.511	0.152	0.064	0.167	0.175	0.110	1	0.2962	**0.0001**	1	**0.0001**
Coprococcus	0.818	0.666	0.406	0.446	0.725	0.066	0.058	0.028	0.082	0.061	**0.0002**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
Pseudobutyrivibrio	0.388	4.477	4.240	3.371	2.865	0.043	0.275	0.201	0.468	0.243	0.2122	1	0.0588	0.764	**0.0001**
Shuttleworthia	0.138	0.121	0.044	0.023	0.013	0.010	0.019	0.008	0.009	0.004	1	**0.0001**	**0.0001**	**0.0001**	**0.0001**
Unclassified *Ruminococcaceae*	5.334	2.515	1.449	1.367	1.548	0.320	0.255	0.141	0.187	0.140	**0.002**	0.0863	**0.0107**	**0.0001**	**0.0001**
Oscillospira	0.304	0.246	0.205	0.125	0.093	0.054	0.022	0.034	0.022	0.024	1	1	1	1	0.0673
Ruminococcus	1.403	1.301	0.584	0.526	0.165	0.132	0.094	0.059	0.259	0.047	**0.0183**	**0.0015**	**0.0001**	**0.0001**	**0.0001**

	Mean relative abundance (%)					SEM[a] (*n* = 8[b])					p-value (compared to no inhibitor at same time (h))				
	Time (h)					Time (h)					Time (h)				
Taxa	**12**	**36**	**60**	**84**	**108**	**12**	**36**	**60**	**84**	**108**	**12**	**36**	**60**	**84**	**108**
Unclassified *Veillonellaceae*	4.760	2.772	0.827	0.439	0.345	0.414	0.114	0.073	0.031	0.036	0.1514	**0.0007**	0.5795	1	**0.0001**
Anaerovibrio	0.341	1.646	2.025	1.263	1.238	0.044	0.090	0.153	0.163	0.165	**0.0314**	**0.0001**	**0.0005**	1	0.1119
Selenomonas	2.447	5.314	1.968	1.217	1.046	0.122	0.243	0.080	0.138	0.057	**0.0023**	**0.0001**	1	0.2492	**0.0001**
Succiniclasticum	2.026	4.056	2.083	1.406	1.522	0.123	0.143	0.118	0.146	0.091	**0.0002**	**0.0001**	**0.0048**	1	**0.0005**
Unclassified [*Mogibacteriaceae*]	0.592	0.413	0.228	0.141	0.127	0.026	0.035	0.036	0.030	0.034	1	0.3058	1	0.348	**0.0001**
Mogibacterium	0.363	0.244	0.154	0.092	0.071	0.042	0.027	0.024	0.006	0.009	**0.03**	0.1891	0.1447	0.0481	**0.0001**
Bulleidia	0.772	0.726	0.295	0.179	0.251	0.054	0.054	0.033	0.027	0.023	0.3468	1	**0.0062**	**0.0001**	**0.0001**
RFN20 *Erysipelotrichaceae*	0.184	0.212	0.345	0.237	0.239	0.019	0.032	0.040	0.039	0.036	**0.0345**	**0.0166**	1	0.1151	**0.0003**
Unclassified *Alphaproteobacteria*	0.060	0.204	0.323	0.409	0.520	0.011	0.034	0.048	0.041	0.048	0.5778	0.0998	0.8648	**0.0002**	**0.0001**
Unclassified *Rickettsiales*	0.191	0.176	0.138	0.072	0.076	0.028	0.021	0.021	0.010	0.021	1	1	1	0.6445	**0.0008**
Unclassified *Succinivibrionaceae*	0.066	0.320	0.184	0.167	0.076	0.014	0.058	0.025	0.075	0.015	0.2829	1	0.4748	**0.0234**	1
SR1 (Bacteria)	0.433	0.135	0.025	0.054	0.430	0.062	0.028	0.007	0.031	0.103	1	1	1	1	**0.0001**
Sphaerochaeta	0.015	0.077	0.167	0.117	0.108	0.006	0.013	0.037	0.017	0.018	1	1	1	**0.0001**	**0.0001**
Treponema	0.978	3.927	3.103	1.638	2.339	0.092	0.283	0.232	0.181	0.299	**0.0143**	**0.0026**	1	**0.0003**	**0.0001**
F16 (TM7)	0.586	0.786	0.068	0.024	0.021	0.054	0.097	0.013	0.008	0.005	1	**0.0001**	0.2143	**0.0001**	**0.0001**
Unclassified *Anaeroplasmataceae*	0.043	0.101	0.162	0.125	0.224	0.014	0.023	0.026	0.034	0.029	1	0.0854	0.618	**0.0002**	0.1403
Anaeroplasma	0.428	0.354	0.243	0.150	0.230	0.034	0.028	0.025	0.015	0.028	**0.0434**	**0.0001**	**0.0003**	**0.0364**	**0.0013**
RF39 (*Mollicutes*)	0.799	0.591	0.374	0.282	0.164	0.069	0.059	0.047	0.044	0.028	0.3604	**0.0001**	**0.0001**	**0.0001**	**0.0001**
Others[e]	1.537	1.236	0.844	0.700	0.786	0.108	0.079	0.074	0.116	0.047	0.5045	0.9832	0.0985	**0.0068**	**0.0001**

[a]Standard error of the mean.

[b]No. of experimental replicates.

[c]Indicates the group contains sequences not classified down to the genus level.

[d]Square brackets indicate suggested but not verified taxon designations;

[e]A small proportion of sequences that were not present at > 0.5% in even one of the samples.

^{13}C-labelled acetate, propionate and butyrate measured in short-term (8 h) assay

The incorporation of $^{13}CO_2$ into ^{13}C-acetate, ^{13}C-propionate and ^{13}C-butyrate was measured using GC-IRMS (SPME; Chapter 2; Section 2.18.5). The ratio of $^{13}C/^{12}C$ was determined for acetate, propionate and butyrate after 8 h of *in vitro* incubation of fresh rumen contents (pre-treatment), and after nine serial transfers in the absence and presence of acetylene. The enrichment data from IRMS combined with the ruminal VFA data (GC-FID) were used to calculate the amount of excess labelled acetate (^{13}A), propionate (^{13}P) and butyrate (^{13}B) present using equations described in Chapter 4. The data summarized in Appendix 2, Table A.2.5 were used to calculate amount of acetate from homoacetogenesis (A_{ha}) and butyrate from homobutyrogenesis (B_{hb}).

Table A.2.5 Mean of ratios of $^{13}C/^{12}C$ and amounts of ^{13}A, ^{13}P and ^{13}B produced in short-term incubations of fresh sheep rumen contents (pre-treatment) and of contents serially-transferred without and with acetylene.

Mean (± SEM[a]) after 8 h of incubation (n = 2[b])							
Pre-treatment							
$NaH^{13}CO_3$ not added							
Inhibitors	**rA_{Uferm}**	**rP_{Uferm}**	**rB_{Uferm}**	**$rCO_{2\ Uferm}$**			
None	0.01112 (± 0.00000)	0.01092 (± 0.00000)	0.01089 (± 0.00001)	0.01088 (± 0.00001)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	**rA_{Lferm}**	**rP_{Lferm}**	**rB_{Lferm}**	**$rCO_{2\ Lferm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.02808 (± 0.00000)	0.01113 (± 0.00006)	0.01524 (± 0.00000)	0.01120 (± 0.000023)	0.31356 (± 0.00514)	2.83100 (± 0.07364)	0.07978 (± 0.00191)
BES	0.02843 (± 0.00002)	0.01138 (± 0.00005)	0.01539 (± 0.00002)	0.01107 (± 0.00009)	0.49083 (± 0.01882)	3.22918 (±0.02842)	0.04843 (± 0.00457)
$CHCl_3$	0.02815 (± 0.00001)	0.01103 (± 0.00003)	0.01536 (± 0.00001)	0.01100 (± 0.00015)	0.11393 (±0.01099)	3.44302 (±0.03472)	0.03237 (± 0.00285)
After serial transfers with no inhibitor							
$NaH^{13}CO_3$ not added							
Inhibitors	**rA_{Uferm}**	**rP_{Uferm}**	**rB_{Uferm}**	**$rCO_{2\ Uferm}$**			
None	0.01114 (± 0.00000)	0.01087 (± 0.00000)	0.01084 (± 0.00000)	0.01080 (± 0.00003)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	**rA_{Lferm}**	**rP_{Lferm}**	**rB_{Lferm}**	**$rCO_{2\ Lferm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.03060 (± 0.00000)	0.01108 (± 0.00001)	0.01459 (± 0.00000)	0.01099 (± 0.00001)	0.21487 (±0.01386)	3.10211 (±0.09099)	0.01745 (± 0.00087)

BES	0.02935 (± 0.00001)	0.01111 (± 0.00004)	0.01456 (± 0.00000)	0.01101 (± 0.00015)	0.21089 (±0.00219)	3.70209 (±0.00957)	0.02902 (± 0.00043)
$CHCl_3$	0.03063 (± 0.00000)	0.01099 (± 0.00000)	0.01460 (± 0.00000)	0.01099 (± 0.00015)	0.10740 (± 0.00175)	3.71667 (± 0.00323)	0.02243 (± 0.00031)

After serial transfers with acetylene

$NaH^{13}CO_3$ not added

Inhibitors	rA_{Uferm}	rP_{Uferm}	rB_{Uferm}	$rCO_{2\ Uferm}$
None	0.01112 (± 0.00000)	0.01086 (± 0.00000)	0.01083 (± 0.00000)	0.01079 (± 0.00001)

$NaH^{13}CO_3$ added (3 mM)

Inhibitors	rA_{Lferm}	rP_{Lferm}	rB_{Lferm}	$rCO_{2\ Lferm}$	^{13}A (µmol/bottle)	^{13}P (µmol/bottle)	^{13}B (µmol/bottle)
None	0.03080 (± 0.00001)	0.01136 (± 0.00001)	0.01449 (± 0.00000)	0.01117 (± 0.000011)	0.37036 (± 0.00107)	4.46133 (± 0.02794)	0.00343 (± 0.00016)
BES	0.03035 (± 0.00012)	0.01126 (± 0.00003)	0.01413 (± 0.00000)	0.01114 (± 0.00009)	0.27886 (± 0.01479)	4.03148 (± 0.21812)	0.00404 (± 0.00197)
$CHCl_3$	0.03043 (± 0.00001)	0.01099 (± 0.00002)	0.01406 (± 0.00001)	0.01111 (± 0.00003)	0.08395 (± 0.01954)	4.16990 (± 0.08509)	0.00602 (± 0.00034)

[a]Standard error of the mean.

[b]No. of experimental replicates.

Appendix 3

Animal diet and gas production data

The sheep weights, amounts of dry matter intake (DMI) and diet refusals by each sheep, methane and carbon dioxide produced from each sheep per day in respiratory chambers are summarised in Table A.3. All sheep used were wethers.

Table A.3.1 Sheep weights, amounts of dry matter intake (DMI), diet refusals, methane and carbon dioxide produced in respiratory chambers for each sheep per day.

Sheep no.	Weight (kg)	Day	DMI (kg/day)	Refusals (kg/day)	Methane (kg/day)	Carbon dioxide (kg/day)
Control sheep						
1	71	1	1.26		0.0313	0.0047
		2	1.16	0.10	0.0301	0.0045
		3	1.26		0.0292	0.0043
		4	1.26		0.0280	0.0041
		5	1.26		0.0287	0.0043
		6	1.26		0.0297	0.0044
		7	1.26		0.0298	0.0043
2	65	1	1.26		0.0304	0.0044
		2	0.68	0.58	0.0276	0.0041
		3	1.03	0.23	0.0217	0.0033
		4	1.26		0.0253	0.0034
		5	1.26		0.0294	0.0036
		6	1.04	0.22	0.0287	0.0041
		7	1.26		0.0277	0.0043
3	68.5	1	1.26		0.0263	0.0042
		2	1.26		0.0293	0.0044
		3	1.26		0.0304	0.0043
		4	1.26		0.0304	0.0042
		5	1.26		0.0260	0.0040
		6	1.26		0.0253	0.0038
		7	1.25	0.01	0.0273	0.0039
4	76.5	1	1.26		0.0287	0.0045
		2	1.26		0.0305	0.0047
		3	1.25	0.01	0.0292	0.0044
		4	1.26		0.0279	0.0041
		5	1.24	0.02	0.0295	0.0044
		6	1.26		0.0316	0.0046
		7	1.26		0.0298	0.0043

Sheep no.	Weight (kg)	Day	DMI (kg/day)	Refusals (kg/day)	Methane (kg/day)	Carbon dioxide (kg/day)
Acetylene-treated sheep						
5	87	1	1.26		0.0301	0.0045
		2	1.26		0.0321	0.0047
		3	1.26		0.0032	0.0045
		4	1.06	0.20	0.0021	0.0042
		5	1.08	0.18	0.0008	0.0035
		6	0.62	0.64	0.0004	0.0030
		7	0.36	0.90	0.0004	0.0027
6	88	1	1.26		0.0259	0.0045
		2	1.26		0.0276	0.0046
		3	1.26		0.0029	0.0043
		4	1.21	0.05	0.0015	0.0040
		5	0.53	0.73	0.0010	0.0035
		6	0.41	0.85	0.0004	0.0032
		7	0.37	0.89	0.0003	0.0028
7	80.5	1	1.26		0.0295	0.0044
		2	1.26		0.0315	0.0046
		3	1.26		0.0013	0.0046
		4	1.24	0.02	0.0004	0.0041
		5	1.24	0.02	0.0002	0.0041
		6	0.85	0.41	0.0002	0.0035
		7	0.59	0.67	0.0002	0.0031
8	82.5	1	1.26		0.0320	0.0048
		2	1.24	0.02	0.0332	0.0049
		3	1.26		0.0016	0.0043
		4	1.19	0.07	0.0003	0.0040
		5	1.14	0.12	0.0002	0.0038
		6	0.94	0.32	0.0002	0.0038
		7	1.16	0.10	0.0002	0.0042
Chloroform-treated sheep						
9	96	1	1.26		0.0329	0.0050
		2	1.26		0.0364	0.0054
		3	1.26		0.0027	0.0052
		4	1.26		0.0027	0.0050
		5	1.26		0.0012	0.0048
		6	1.26		0.0007	0.0047
		7	1.26		0.0005	0.0048
10	83	1	1.26		0.0317	0.0052
		2	1.26		0.0339	0.0052
		3	1.26		0.0023	0.0048
		4	1.26		0.0013	0.0045
		5	1.26		0.0005	0.0042
		6	1.26		0.0009	0.0046
		7	1.26		0.0004	0.0050

Sheep no.	Weight (kg)	Day	DMI (kg/day)	Refusals (kg/day)	Methane (kg/day)	Carbon dioxide (kg/day)
11	97.5	1	1.26		0.0356	0.0054
		2	1.26		0.0363	0.0054
		3	1.26		0.0025	0.0051
		4	1.26		0.0019	0.0047
		5	1.26		0.0011	0.0045
		6	1.26		0.0018	0.0052
		7	1.26		0.0018	0.0053
12†	83	1	1.26		0.0259	0.0050
		2	1.26		0.0247	0.0048
		3	1.26		0.0024	0.0047
		4	1.26		0.0015	0.0045
		5	0.04	1.22	0.0009	0.0031
		6	0.05	1.21	0.0008	0.0028

† Sheep removed from the trial due to refusal of diet and health issues.

Table A.3.2 Mean concentrations and proportions of products in the control, acetylene- and chloroform-treated sheep over time. The values are the concentrations in rumen samples collected at approximately 9 a.m. on the day noted.

	Control sheep							Acetylene-treated sheep							Chloroform-treated sheep						
	Day							Day							Day						
Products in mM	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Acetate	50.59	56.06	57.26	56.01	60.18	51.27	45.86	47.82	56.42	45.06	55.63	55.57	44.81	29.86	42.48	46.62	33.80	46.64	41.10	37.56	36.06
Propionate	11.10	13.07	13.07	12.27	13.79	12.36	11.17	10.69	13.80	25.55	27.57	21.15	17.94	13.54	9.48	10.49	18.21	27.16	24.20	19.85	18.50
Butyrate	12.02	12.70	13.31	13.35	15.18	12.63	11.11	10.86	12.80	16.14	19.39	15.53	11.54	7.12	10.28	11.67	14.06	18.01	13.94	10.98	10.70
Isobutyrate	0.98	1.07	1.06	0.99	1.04	1.09	1.10	1.03	1.16	1.08	0.93	0.85	0.90	0.78	1.07	1.07	0.88	1.06	1.17	1.03	1.12
Isovalerate	1.04	1.22	1.21	1.06	1.17	1.30	1.30	1.18	1.43	1.24	1.04	0.91	0.97	0.80	1.30	1.42	1.11	1.72	1.74	1.55	1.61
Valerate	0.66	0.85	0.85	0.77	0.94	0.83	0.75	0.64	0.95	1.29	2.14	1.25	1.17	0.81	0.68	0.78	1.01	1.85	2.72	1.34	1.23
Caproate	0.28	0.34	0.38	0.31	0.36	0.29	0.23	0.25	0.38	0.05	0.23	0.06	0.06	0.05	0.28	0.33	0.06	0.16	0.58	0.32	0.30
Lactate	0.12	0.06	0.08	0.05	0.11	0.08	<0.01	0.04	0.06	<0.01	<0.01	0.10	0.32	<0.01	<0.01	<0.01	<0.01	<0.01	0.77	<0.01	<0.01
Formate	0.43	0.49	0.16	0.16	<0.01	<0.01	<0.01	0.63	0.54	5.06	1.16	0.36	1.30	2.57	<0.01	0.37	6.06	4.20	6.43	4.81	4.67
Ethanol	5.60	9.51	10.89	5.33	3.66	4.69	5.85	3.65	12.28	6.99	4.66	1.95	2.82	3.76	4.48	3.93	4.69	2.96	<0.01	5.41	6.77
Total VFA	76.68	85.31	87.14	84.76	92.67	79.78	71.51	72.47	86.94	90.41	106.9	95.33	77.38	52.97	65.56	72.39	69.12	96.60	85.45	72.62	69.52
Proportion																					
Acetate	0.66	0.66	0.66	0.66	0.65	0.64	0.64	0.66	0.65	0.50	0.53	0.58	0.58	0.57	0.65	0.65	0.49	0.49	0.49	0.52	0.52
Propionate	0.14	0.15	0.15	0.15	0.15	0.16	0.16	0.15	0.16	0.28	0.26	0.22	0.23	0.25	0.14	0.14	0.26	0.28	0.29	0.27	0.27
Butyrate	0.16	0.15	0.15	0.16	0.16	0.16	0.15	0.15	0.15	0.18	0.18	0.16	0.15	0.14	0.16	0.16	0.20	0.18	0.15	0.15	0.15
Acetate: propionate	4.56	4.30	4.38	4.55	4.30	4.16	4.10	4.62	4.16	1.77	2.06	2.64	2.61	2.37	4.48	4.46	1.87	1.76	1.71	1.90	1.96
(SEM[a]; n = 4 or 3[b])																					
Acetate	4.65	6.26	5.67	5.70	10.59	3.54	3.33	3.15	6.37	4.30	4.46	2.72	2.17	4.14	2.07	4.37	2.59	8.48	7.60	1.90	1.78
Propionate	0.86	1.44	1.02	1.37	3.23	1.34	1.13	0.25	1.17	2.14	3.99	1.55	1.37	1.07	0.08	1.32	1.52	5.62	4.55	2.09	1.73
Butyrate	1.03	1.51	1.23	0.87	1.83	0.53	0.53	1.56	2.14	2.56	3.49	1.37	2.58	3.01	0.37	1.13	1.87	6.34	4.77	1.29	1.13
Isobutyrate	0.06	0.09	0.09	0.04	0.09	0.09	0.05	0.08	0.08	0.05	0.05	0.07	0.09	0.11	0.14	0.17	0.11	0.18	0.21	0.14	0.10
Isovalerate	0.11	0.11	0.13	0.04	0.10	0.14	0.08	0.14	0.16	0.11	0.14	0.12	0.15	0.16	0.26	0.33	0.30	0.64	0.53	0.44	0.37
Valerate	0.04	0.06	0.08	0.05	0.11	0.06	0.03	0.04	0.08	0.02	0.20	0.02	0.02	0.04	0.07	0.09	0.02	0.08	0.47	0.15	0.14

	Control sheep							Acetylene-treated sheep							Chloroform-treated sheep						
	Day							Day							Day						
Products in mM	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Caproate	0.07	0.13	0.13	0.09	0.22	0.11	0.07	0.12	0.18	0.29	0.96	0.18	0.18	0.22	0.10	0.17	0.22	0.70	1.51	0.32	0.24
Lactate	0.10	0.05	0.12	0.04	0.08	0.08	-	0.04	0.05	-	-	0.09	0.20	-	-	-	-	-	0.77	1	-
Formate	0.38	0.43	0.16	0.16	-	-	-	0.37	0.33	0.63	0.33	0.32	0.83	0.90	-	0.37	0.62	0.63	0.75	0.59	0.63
Ethanol	4.41	5.48	4.95	0.92	1.55	1.63	1.17	1.30	7.53	2.35	1.37	1.19	1.65	2.19	1.07	1.64	1.88	2.19	-	2.74	1.55
Total VFA	6.53	9.45	8.28	7.95	16.09	5.31	5.01	5.16	10.05	9.30	12.96	5.57	5.72	8.45	2.76	7.52	6.34	21.85	19.37	6.11	4.86
Proportion																					
Acetate	0.01	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.02	0.01	0.02	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.01
Propionate	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.02	0.02	0.02	0.02
Butyrate	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.36	0.15	0.06	0.12	0.04	0.28	0.31	0.14	0.12	0.10	0.11	0.02	0.05	0.13
Acetate: propionate	0.06	0.10	0.08	0.23	0.18	0.28	0.16	0.01	0.01	0.01	0.01	0.00	0.02	0.02	0.00	0.00	0.01	0.01	0.01	0.01	0.02
p-value compared to day 1 of each animal/treatment group																					
Acetate		1	1	1	1	1	1		1	1	1	1	1	0.0175		1	0.1733	1	1	1	1
Propionate		1	1	1	1	1	1		1	0.0001	0.0001	0.006	0.288	1		1	0.0265	0.0001	0.0009	0.0362	0.1326
Butyrate		1	1	1	1	1	1		1	0.9038	0.0776	1	1	1		1	1	0.8057	1	1	1
Isobutyrate		1	1	1	1	1	1		1	1	1	1	1	0.8952		1	1	1	1	1	1
Isovalerate		1	1	1	1	1	1		1	1	1	1	1	0.904		1	1	1	1	1	1
Valerate		1	1	1	1	1	1		1	1	0.0594	1	1	1		1	1	0.8662	0.1006	1	1
Caproate		1	1	1	1	1	1		1	0.2036	1	0.9383	0.9111	0.3554		1	0.6579	1	1	1	1
Lactate		1	1	1	1	1	1		1	1	1	1	1	1		1	1	1	0.7984	1	1
Formate		1	1	1	1	1	1		1	0.0001	1	1	1	0.6531		1	0.0001	0.0001	0.0001	0.0001	0.0001
Ethanol		1	1	1	1	1	1		1	1	1	1	1	1		1	1	1	1	1	1
Total VFA		1	1	1	1	1	1		1	1	0.2444	1	1	1		1	1	1	1	1	1
Proportion																					
Acetate		1	1	1	1	1	1		1	0.0001	0.0001	0.0028	0.0027	0.0002		1	0.0001	0.0001	0.0001	0.0001	0.0001
Propionate		1	1	1	1	1	1		1	0.0001	0.0001	0.0001	0.0001	0.0001		1	0.0001	0.0001	0.0001	0.0001	0.0001
Butyrate		1	1	1	1	1	1		1	0.8093	1	1	1	1		1	0.0357	1	1	1	1
Acetate:		1	1	1	1	1	1		1	0.0001	0.0001	0.0001	0.0001	0.0001		1	0.0001	0.0001	0.0001	0.0001	0.0001

	Control sheep							Acetylene-treated sheep							Chloroform-treated sheep						
	Day							Day							Day						
Products in mM	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Propionate																					

[a]Standard error of the mean.

[b]No. of sheep (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).

Table A.3.3 Mean amounts of methane and dry matter intake (DMI) of control, acetylene- and chloroform-treated sheep over time. The methane yields are calculated from the amount of methane emitted in the preceding 24 h (approximately 9 a.m. to 9 a.m.), and the amount of feed consumed in the same period.

	Control sheep							Acetylene-treated sheep							Chloroform-treated sheep						
	Day							Day							Day						
	2	**3**	**4**	**5**	**6**	**7**	**8**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Methane (g/kg DMI)	23.17	28.53	22.92	22.15	22.62	24.06	22.80	23.32	24.81	1.78	0.98	0.74	0.53	0.61	26.51	28.23	1.98	1.57	0.74	0.92	0.70
DMI (kg/day)	1.26	1.09	1.20	1.26	1.26	1.21	1.26	1.26	1.25	1.26	1.17	1.00	0.71	0.62	1.26	1.26	1.26	1.26	1.26	1.26	1.26
(SEM[a]; $n = 4$ or 3[b])																					
Methane (g/kg DMI)	0.87	4.10	0.68	0.83	0.68	1.56	0.50	1.02	1.06	0.37	0.41	0.42	0.19	0.21	0.91	0.65	0.10	0.33	0.16	0.27	0.36
DMI (kg/day)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.04	0.16	0.12	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00
p-value compared to day 1 of each animal/treatment group																					
Methane (g/kg DMI)		1	1	1	1	1	1		1	0.0001	0.0001	0.0001	0.0001	0.0001		1	0.0001	0.0001	0.0001	0.0001	0.0001
DMI (kg/day)		1	1	1	1	1	1		1	1	1	1	0.007	0.0003		-	-	-	-	-	-

[a]Standard error of the mean.

[b]No. of sheep (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).

Table A.3.4 The number of reads and OTUs for each sample sequence for archaea and bacteria in the rumen contents of control, acetylene- and chloroform-treated sheep over time.

		Archaea		Bacteria	
Sheep no.	**Day**	**No. of reads**	**No. of OTUs**	**No. of reads**	**No. of OTUs**
Control sheep					
1	1	669	48	8221	4184
	3	1033	55	5166	3130
	4	1161	48	5256	2759
	5	1213	39	3617	2041
	6	1333	47	5468	2748
	7	3159	68	4089	2067
	8	1966	58	5644	2842
2	1	575	43	2769	1581
	3	550	38	1464	917
	4	337	28	2793	1600
	5	726	34	1078	723
	6	673	40	4791	2530
	7	668	40	3968	1973
	8	646	36	5225	2590
3	1	1119	32	10250	4534
	3				
	4	4104	100	3142	1742
	5	1120	47	3006	1679
	6	766	31	4488	2360
	7	933	37	3917	2230
	8	999	45	4784	2534
4	1	292	23	2489	1510
	3	1078	48	4540	2523
	4	703	39	3097	1743
	5	1737	47	5090	2622
	6	734	36	7094	3518
	7	1099	40	5738	2534
	8	2190	47	5340	2814
Acetylene-treated sheep					
5	1	1208	33	5346	2900
	3				
	4	1635	26	5213	2694
	5	3129	45	7212	3536
	6	1693	30	9275	4412
	7	1512	27	9500	4485
	8	1071	19	563	363
6	1	1073	48	11111	5380
	3	1716	50	9433	5095
	4	967	42	7864	3711
	5	1662	32	387	290
	6	1432	34	4349	2273
	7	1511	34	2553	1417
	8	1218	21	6125	2707

		Archaea		Bacteria	
Sheep no.	**Day**	**No. of reads**	**No. of OTUs**	**No. of reads**	**No. of OTUs**
7	1	1080	66	6831	4097
	3				
	4	2591	48	17337	5772
	5	1087	34	3467	1723
	6	1049	16	5309	2503
	7	1750	18	7795	3636
	8	964	26	9411	4028
8	1	1075	47	5395	2712
	3	874	45	2914	1627
	4	1014	43	4819	2225
	5	1358	21	13403	4539
	6	1730	35	7150	3408
	7	1128	21	7469	3223
	8	1410	33	10904	4522
Chloroform-treated sheep					
9	1	1506	63	5550	3044
	3				
	4	2065	66	9675	5175
	5	1801	43	14353	5534
	6	1038	34	4176	2074
	7	1554	38	5202	2645
	8	1430	13	5901	2604
10	1	1264	54	3701	2199
	3	1161	43	10165	4735
	4	1631	56	11566	4478
	5	1778	47	17462	5984
	6	2685	35	8644	3471
	7	1457	20	6314	2624
	8	1276	42	3932	1890
11	1	1395	59	5315	2698
	3	1145	58	10973	5151
	4	1258	59	8052	3600
	5	2058	47	10858	4417
	6	2033	50	8019	3385
	7	2601	37	12482	5298
	8	1997	22	7980	3132
12†	1	1724	60	5190	2278
	3	1703	56	4764	2165
	4	2050	54	5584	2439
	5	2320	78	6481	2768
	6	2024	76	10302	3958
	7	2717	98	10807	3885

† Sheep removed from the trial due to refusal of diet and health issues.

Table A.3.5 The most abundant and prevalent rumen bacterial genus-level groups in the rumen contents of control sheep over time. The 12 most abundant groups (mean relative abundance > 1% in any one sample) are indicated in bold font and the sequences which have significant p-value < 0.05 are also indicated in bold font.

	Mean relative abundance (%)							**SEM[a] (*n* = 4[b])**							**p-value compared to day 1**						
	Day							**Day**							**Day**						
Taxa	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Corynebacterium	0.006	0.052	0.029	0.026	0.055	0.006	0.005	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
Unclassified[c]																					
Coriobacteriaceae	0.266	0.285	0.273	0.301	0.411	0.302	0.268	<0.001	0.001	0.001	0.001	0.001	<0.001	0.001		1	1	1	1	1	1
Unclassified																					
Bacteroidales	12.04	11.51	11.44	11.34	11.23	10.88	10.18	0.009	0.029	0.016	0.019	0.013	0.011	0.011		1	1	1	1	1	1
BS11 (*Bacteroidales*)	1.303	1.441	1.119	1.046	0.793	1.092	0.836	0.002	0.004	0.003	0.001	0.001	0.002	0.001		1	1	1	1	1	1
BF311 (*Bacteroidaceae*)	0.548	0.552	0.763	0.640	0.549	0.624	0.358	0.002	0.001	0.002	0.001	<0.001	0.001	0.001		1	1	1	1	1	1
Paludibacter	0.103	0.056	0.088	0.090	0.065	0.059	0.062	0.001	<0.001	0.001	0.001	0.001	<0.001	<0.001		1	1	1	1	1	1
Unclassified																					
Prevotellaceae	0.624	0.278	0.422	0.353	0.550	0.487	0.543	0.002	<0.001	0.001	0.001	0.001	0.002	0.001		1	1	1	1	1	1
Prevotella	22.61	20.51	20.61	21.34	23.87	25.30	26.05	0.015	0.022	0.012	0.009	0.015	0.019	0.036		1	1	1	1	1	1
RF16 (*Bacteroidales*)	1.082	1.011	0.914	0.698	1.115	2.570	1.335	0.003	0.001	0.002	0.002	0.003	0.017	0.005		1	1	1	1	1	1
S24-7 (*Bacteroidales*)	1.881	2.498	2.484	2.351	2.062	1.805	2.158	0.004	0.006	0.005	0.006	0.002	0.003	0.003		1	1	1	1	1	1
Unclassified																					
[*Paraprevotellaceae*][d]	0.523	0.354	0.611	0.676	0.603	0.604	0.552	0.001	0.001	0.003	0.002	0.002	0.001	0.001		1	1	1	1	1	1
CF231																					
[*Paraprevotellaceae*]	0.728	0.722	0.782	0.716	0.802	0.805	0.665	0.002	0.002	0.001	0.001	0.001	0.001	0.001		1	1	1	1	1	1
YRC22																					
[*Paraprevotellaceae*]	0.886	0.802	0.901	0.746	0.664	0.984	0.840	0.003	0.002	0.001	0.001	0.001	0.001	0.001		1	1	1	1	1	1
[*Prevotella*]																					
[*Paraprevotellaceae*]	0.027	0.026	0.043	0.010	0.061	0.042	0.028	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
p-2534-18B5																					
(*Bacteroidales*)	0.211	0.465	0.430	0.960	1.224	3.358	0.983	0.001	0.005	0.002	0.008	0.007	0.028	0.006		1	1	1	1	1	1
SHD-231																					
(*Anaerolinaceae*)	0.546	0.233	0.289	0.254	0.313	0.306	0.211	0.002	0.001	0.001	0.001	0.001	0.001	0.001		0.0097	0.4305	0.1024	1	1	**0.0164**
YS2 (*Cyanobacteria*)	0.239	0.299	0.247	0.376	0.380	0.316	0.330	0.001	0.001	0.001	0.001	0.001	0.001	<0.001		1	1	0.9838	0.8253	1	1
Fibrobacter	5.414	4.977	6.755	8.044	6.321	6.395	6.011	0.014	0.009	0.026	0.028	0.012	0.007	0.012		1	1	1	1	1	1
Lactobacillus	0.003	0.029	0.005	<0.001	0.014	0.004	0.005	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
Streptococcus	0.060	0.105	0.092	0.186	0.098	0.085	0.160	<0.001	0.001	<0.001	0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1

	Mean relative abundance (%)							SEM[a] (*n* = 4[b])							p-value compared to day 1						
	Day							Day							Day						
Taxa	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Unclassified																					
Clostridiales	12.60	12.72	12.29	11.81	10.61	9.91	10.21	0.011	0.001	0.006	0.014	0.009	0.008	0.004	1	1	1	1	1	1	
Unclassified																					
Christensenellaceae	0.155	0.197	0.175	0.113	0.135	0.142	0.093	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Clostridium	0.707	0.748	0.793	0.949	1.015	0.567	0.525	0.001	0.001	0.002	0.003	0.001	0.001	0.001	1	1	1	1	1	1	
Unclassified																					
Lachnospiraceae	8.474	10.09	8.858	9.240	7.789	6.458	7.543	0.011	0.012	0.012	0.013	0.008	0.008	0.002	1	1	1	1	1	1	
Anaerostipes	0.071	0.021	0.062	0.098	0.041	0.011	0.050	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Blautia	0.562	0.449	0.499	0.546	0.467	0.401	0.468	0.001	0.001	0.001	0.002	0.002	0.002	0.001	1	1	1	1	1	1	
Butyrivibrio	3.272	4.339	3.272	3.843	3.877	3.160	4.391	0.005	0.009	0.009	0.005	0.006	0.003	0.006	1	1	1	1	1	1	
Coprococcus	0.921	0.892	0.986	1.006	1.263	0.959	1.046	0.001	<0.001	0.001	0.001	0.002	0.002	0.001	1	1	1	1	1	1	
Moryella	0.513	0.594	0.452	0.479	0.471	0.351	0.554	0.001	0.001	0.001	<0.001	0.001	<0.001	0.001	1	1	1	1	1	1	
Pseudobutyrivibrio	0.793	0.737	0.960	1.281	0.935	0.842	1.194	0.001	0.002	0.003	0.002	0.002	0.002	0.002	1	1	1	1	1	1	
Unclassified																					
Ruminococcaceae	7.023	8.207	7.153	6.262	6.467	6.167	5.869	0.008	0.006	0.005	0.002	0.005	0.003	0.005	1	1	1	1	1	1	
Ruminococcus	5.828	5.668	5.963	5.312	5.506	5.677	6.658	0.007	0.015	0.011	0.005	0.005	0.004	0.004	1	1	1	1	1	1	
Unclassified																					
Veillonellaceae	1.081	0.778	0.804	0.892	1.367	1.255	1.265	0.003	0.002	0.001	0.003	0.003	0.004	0.003	1	1	1	1	1	1	
Selenomonas	0.255	0.191	0.267	0.247	0.306	0.282	0.449	0.001	<0.001	<0.001	0.001	0.001	0.001	<0.001	1	1	1	1	1	1	
Succiniclasticum	1.188	1.537	1.660	1.498	1.522	1.329	1.409	0.001	0.002	0.001	0.002	0.001	0.003	0.003	1	1	1	1	1	1	
Unclassified																					
[*Mogibacteriaceae*]	0.844	0.735	0.737	0.552	0.723	0.651	0.707	0.002	<0.001	<0.001	0.002	0.001	0.002	0.001	1	1	1	1	1	1	
Mogibacterium	0.894	0.908	0.793	0.829	0.677	0.630	0.523	0.002	0.002	0.001	0.001	0.001	0.001	0.001	1	1	1	1	1	0.998	
Bulleidia	0.425	0.405	0.443	0.316	0.460	0.358	0.574	0.001	0.001	0.001	0.001	0.001	0.001	0.001	1	1	1	1	1	1	
RFN20																					
Erysipelotrichaceae	0.640	0.381	0.443	0.219	0.366	0.379	0.427	0.001	0.001	<0.001	0.001	<0.001	<0.001	0.001	1	1	0.0728	1	1	1	
Unclassified																					
Alphaproteobacteria	0.072	0.040	0.097	0.073	0.031	0.046	0.071	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Unclassified *Rickettsiales*	0.128	0.063	0.130	0.115	0.110	0.166	0.038	<0.001	<0.001	0.001	<0.001	<0.001	0.001	<0.001	1	1	1	1	1	1	
Unclassified																					
Succinivibrionaceae	0.171	0.052	0.114	0.120	0.253	0.199	0.112	0.001	<0.001	<0.001	0.001	0.001	0.001	<0.001	1	1	1	1	1	1	
Treponema	1.301	1.147	1.834	1.394	1.337	1.031	1.300	<0.001	0.001	0.005	0.004	0.003	0.002	0.003	1	1	1	1	1	1	
TG5																					
(*Dethiosulfovibrionaceae*)	0.037	<0.001	0.017	0.064	0.060	0.063	0.089	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	1	1	1	1	1	1	
F16 (TM7)	0.323	0.256	0.188	0.141	0.211	0.205	0.107	0.001	0.001	0.001	0.001	<0.001	0.001	<0.001	1	1	1	1	1	1	
Unclassified																					
Anaeroplasmataceae	0.019	0.013	0.005	0.053	0.016	0.034	0.029	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Anaeroplasma	0.386	0.120	0.394	0.272	0.341	0.328	0.303	<0.001	<0.001	0.001	0.001	0.001	<0.001	<0.001	0.4202	1	1	1	1	1	

	Mean relative abundance (%)							SEM[a] (n = 4[b])							p-value compared to day 1						
	Day							Day							Day						
Taxa	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
RF39 (*Mollicutes*)	0.488	0.630	0.747	0.486	0.686	0.465	0.647	0.001	0.001	0.001	0.001	0.002	0.001	0.002		1	1	1	1	1	1
Others[e]	1.733	1.885	1.561	1.641	1.790	1.921	1.770	0.002	0.001	0.001	0.001	0.002	0.001	0.001		1	1	1	1	1	1

[a]Standard error of the mean.

[b]No. of sheep.

[c]Indicates the group contains sequences not classified down to the genus level.

[d]Square brackets indicate suggested but not verified taxon designations;

[e]A small proportion of sequences that were not present at > 0.5% in even one of the samples.

Table A.3.6 The most abundant and prevalent rumen bacterial genus-level groups in the rumen contents of acetylene-sheep over time. The 12 most abundant groups (mean relative abundance > 1% in any one sample) are indicated in bold font and the sequences which have significant p-value < 0.05 are also indicated in bold font.

	Mean relative abundance (%)							SEM[a] (n = 4[b])							p-value compared to day 1						
	Day							Day							Day						
Taxa	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Corynebacterium	0.009	0.005	0.008	0.031	0.021	0.022	0.136	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001		1	1	1	1	1	1
Unclassified[c]																					
Coriobacteriaceae	0.223	0.242	0.109	0.049	0.140	0.103	0.040	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	**0.0031**	1	0.6457	**0.0007**
Unclassified																					
Bacteroidales	9.67	9.96	10.74	12.32	13.91	14.36	12.45	0.013	0.023	0.013	0.018	0.007	0.010	0.010		1	1	1	0.1019	0.0601	1
BS11 (*Bacteroidales*)	1.361	0.827	0.243	0.092	0.139	0.202	0.196	0.004	0.002	0.001	<0.001	0.001	0.001	0.001		1	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0004**
BF311 (*Bacteroidaceae*)	0.924	0.730	1.082	1.007	1.109	1.110	0.801	0.002	0.001	0.003	0.003	0.003	0.002	0.002		1	1	1	1	1	1
Paludibacter	0.065	0.110	0.206	<0.001	0.121	0.216	0.524	<0.001	<0.001	0.002	<0.001	0.001	0.001	0.003		1	1	1	1	1	0.969

	Mean relative abundance (%)							SEM[a] (*n* = 4[b])							p-value compared to day 1						
	Day							Day							Day						
Taxa	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Unclassified *Prevotellaceae*	0.571	0.648	0.885	0.851	0.615	0.674	0.464	0.002	0.002	0.001	0.002	0.002	0.001	0.002		1	1	1	1	1	1
Prevotella	22.69	23.40	36.16	37.22	31.62	30.82	31.06	0.014	0.027	0.017	0.013	0.018	0.031	0.030		1	**0.0001**	**0.0001**	**0.0288**	0.1525	0.1646
RF16 (*Bacteroidales*)	1.553	0.488	0.987	1.168	1.763	2.459	2.625	0.003	0.002	0.003	0.004	0.005	0.010	0.010		0.4501	1	1	1	1	1
S24-7 (*Bacteroidales*)	2.761	2.744	2.820	2.175	1.851	1.808	1.879	0.002	<0.001	0.003	0.002	0.004	0.003	0.004		1	1	1	1	1	1
Unclassified [*Paraprevotellaceae*][d]	0.516	0.413	0.478	0.443	0.559	0.414	0.480	0.001	<0.001	<0.001	0.001	0.001	<0.001	0.001		1	1	1	1	1	1
CF231 [*Paraprevotellaceae*]	0.872	0.780	0.867	0.694	1.000	1.031	1.538	0.001	0.002	0.002	0.002	0.001	0.002	0.005		1	1	1	1	1	1
YRC22 [*Paraprevotellaceae*] [*Prevotella*]	0.632	0.667	0.546	0.692	0.625	0.793	0.897	0.001	0.001	0.001	0.001	0.001	0.002	0.002		1	1	1	1	1	1
[*Paraprevotellaceae*] p-2534-18B5	0.034	0.058	0.045	0.036	0.070	0.170	0.335	<0.001	0.001	<0.001	<0.001	<0.001	0.001	0.002		1	1	1	1	0.9747	0.0121
(*Bacteroidales*) SHD-231	0.002	0.005	<0.001	0.009	0.010	0.028	1.034	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.010		1	1	1	1	1	1
(*Anaerolinaceae*)	0.183	0.188	0.053	0.011	0.018	0.018	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	**0.0411**	**0.0001**	**0.0002**	**0.0003**	**0.0001**
YS2 (*Cyanobacteria*)	0.476	0.296	0.130	0.066	0.107	0.059	0.135	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0003**
Fibrobacter	5.911	3.920	11.51	7.656	7.277	6.666	8.640	0.017	0.005	0.005	0.012	0.014	0.020	0.031		1	0.0872	1	1	1	1
Lactobacillus	0.058	0.037	0.006	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
Streptococcus	0.037	0.144	0.134	0.073	0.050	0.044	0.011	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
Unclassified *Clostridiales*	13.91	14.08	6.77	7.78	8.88	8.41	7.75	0.012	0.002	0.007	0.008	0.004	0.003	0.011		1	**0.0001**	**0.0001**	**0.0091**	**0.0026**	**0.0002**
Unclassified *Christensenellaceae*	0.233	0.127	0.047	0.016	0.068	0.105	0.049	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	**0.0407**	**0.007**	0.9362	1	0.5945
Clostridium	0.693	1.070	0.137	0.107	0.069	0.119	0.170	<0.001	0.002	<0.001	0.001	<0.001	0.001	0.001		1	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.002**
Unclassified *Lachnospiraceae*	9.69	11.84	6.84	5.99	6.57	6.13	5.11	0.007	0.047	0.012	0.007	0.004	0.006	0.006		1	**0.0121**	**0.0079**	0.1962	0.0788	**0.0033**
Anaerostipes	0.034	0.037	0.026	0.081	0.016	0.016	0.031	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
Blautia	0.196	0.198	0.183	0.128	0.186	0.263	0.255	<0.001	<0.001	<0.001	0.001	<0.001	0.001	0.001		1	1	1	1	1	1
Butyrivibrio	2.93	3.23	3.87	4.93	5.05	5.15	3.99	0.004	0.009	0.005	0.014	0.008	0.011	0.009		1	1	1	1	1	1
Coprococcus	1.187	1.427	0.667	0.877	0.918	0.602	0.532	0.002	0.001	0.001	0.001	0.001	0.001	0.002		1	1	1	1	0.9342	0.0916
Moryella	0.377	0.213	0.301	0.266	0.347	0.302	0.219	0.001	0.001	<0.001	<0.001	<0.001	0.001	0.001		1	1	1	1	1	1
Pseudobutyrivibrio	0.762	1.065	0.489	0.378	0.496	0.419	0.257	0.001	0.004	<0.001	0.001	0.001	0.001	0.001		1	1	1	1	1	1
Unclassified *Ruminococcaceae*	6.210	6.969	2.407	2.322	3.460	3.759	3.944	0.001	0.002	0.003	0.003	0.006	0.002	0.007		1	**0.0001**	**0.0001**	**0.0001**	**0.0003**	**0.0007**
Ruminococcus	5.846	4.978	1.880	2.400	2.460	2.312	2.297	0.013	0.021	0.006	0.003	0.002	0.003	0.006		1	**0.0001**	0.052	0.1646	0.1209	0.089

	Mean relative abundance (%)							SEM[a] (*n* = 4[b])							p-value compared to day 1						
	Day							Day							Day						
Taxa	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Unclassified *Veillonellaceae*	0.589	0.507	0.687	0.842	1.120	2.120	2.428	0.002	0.003	0.002	0.002	0.003	0.012	0.013	1	1	1	1	0.4458	0.273	
Selenomonas	0.358	0.373	0.742	1.010	0.702	0.350	0.487	0.001	0.002	0.001	0.001	0.001	0.001	0.001	1	0.287	**0.0069**	0.7802	1	1	
Succiniclasticum	1.268	1.380	1.986	1.977	2.113	2.546	2.037	0.001	0.001	0.001	0.001	0.002	0.002	0.002	1	0.1055	0.3111	0.1157	**0.0023**	0.2889	
Unclassified [*Mogibacteriaceae*]	0.902	0.746	0.623	0.699	0.565	0.631	0.589	0.001	0.001	0.002	0.001	0.001	0.001	<0.001	1	1	1	1	1	1	
Mogibacterium	0.914	0.859	0.608	0.441	0.470	0.503	0.279	0.001	0.002	0.001	0.001	0.001	0.001	0.001	1	0.5674	**0.0397**	0.1916	0.5273	**0.0011**	
Bulleidia	0.561	0.492	0.394	0.397	0.411	0.435	0.355	0.001	0.002	<0.001	0.001	0.001	0.001	0.001	1	1	1	1	1	1	
RFN20 *Erysipelotrichaceae*	0.450	0.210	0.140	0.081	0.167	0.162	0.140	0.001	0.001	<0.001	<0.001	<0.001	0.001	0.001	1	0.1342	**0.0012**	0.3238	0.1121	**0.0159**	
Unclassified *Alphaproteobacteria*	0.101	0.037	0.005	0.121	0.240	0.473	1.668	<0.001	<0.001	<0.001	0.001	0.001	0.002	0.008	1	0.4384	1	1	1	1	
Unclassified *Rickettsiales*	0.202	0.057	0.080	0.044	0.076	0.097	0.208	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	1	1	1	1	1	1	
Unclassified *Succinivibrionaceae*	0.102	0.124	0.275	0.227	0.103	0.092	0.076	<0.001	0.001	0.002	0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Treponema	0.921	1.205	2.155	2.452	2.278	1.650	1.273	0.001	<0.001	0.004	0.005	0.006	0.005	0.005	1	**0.0296**	**0.0085**	**0.044**	1	1	
TG5 (*Dethiosulfovibrionaceae*)	0.020	0.028	0.016	0.014	0.025	0.072	0.233	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	1	1	1	1	1	0.5111	
F16 (TM7)	0.202	0.482	0.137	0.051	0.220	0.168	0.134	0.001	0.003	0.001	<0.001	0.001	0.001	0.001	1	1	0.4462	1	1	1	
Unclassified *Anaeroplasmataceae*	0.011	0.005	0.031	0.136	0.008	0.006	0.002	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Anaeroplasma	0.279	0.282	0.179	0.098	0.160	0.093	0.111	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.001	1	1	0.4164	1	0.5687	0.6764	
RF39 (*Mollicutes*)	0.579	0.549	0.131	0.218	0.273	0.414	0.525	0.001	<0.001	<0.001	0.001	0.001	0.001	0.001	1	**0.0135**	0.2769	1	1	1	
Others[e]	1.921	1.767	1.189	1.319	1.548	1.591	1.610	0.001	0.003	<0.001	0.001	0.001	0.001	0.001	1	**0.0101**	0.0672	1	1	1	

[a]Standard error of the mean.

[b]No. of sheep.

[c]Indicates the group contains sequences not classified down to the genus level.

[d]Square brackets indicate suggested but not verified taxon designations;

[e]A small proportion of sequences that were not present at > 0.5% in even one of the samples.

Table A.3.7 The most abundant and prevalent rumen bacterial genus-level groups in the rumen contents of chloroform-treated sheep over time. The 13 most abundant groups (mean relative abundance > 1% in any one sample) are indicated in bold font and the sequences which have significant p-value < 0.05 are also indicated in bold font.

	Mean relative abundance (%)							**SEM[a] (*n* = 3[b])**							**p-value compared to day 1**						
	Day							**Day**							**Day**						
Day	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Corynebacterium	0.009	0.044	0.017	<0.001	0.008	0.005	0.006	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
Unclassified[c] *Coriobacteriaceae*	0.215	0.080	0.085	0.043	0.072	0.037	0.055	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	0.8164	**0.0291**	0.347	**0.0147**	0.1061
Unclassified *Bacteroidales*	11.58	10.30	13.15	12.86	10.46	12.28	11.89	0.021	0.011	0.016	0.012	0.006	0.006	0.007		1	1	1	1	1	1
BS11 (*Bacteroidales*)	1.801	1.144	0.122	0.123	0.136	0.086	0.034	0.003	0.005	<0.001	<0.001	0.001	<0.001	<0.001		1	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
BF311 (*Bacteroidaceae*)	0.230	0.472	0.715	0.743	0.920	0.927	0.769	0.002	0.004	0.003	0.002	0.001	0.001	0.001		1	0.3218	0.3971	0.0994	0.1283	0.5387
Paludibacter	0.047	0.014	0.086	0.092	0.182	0.407	0.388	<0.001	<0.001	<0.001	<0.001	0.001	0.003	0.002		1	1	1	1	1	1
Unclassified *Prevotellaceae*	0.268	0.260	0.494	0.441	0.466	0.326	0.259	0.001	<0.001	0.001	<0.001	0.001	<0.001	<0.001		1	1	1	1	1	1
Prevotella	20.13	20.40	29.65	33.46	34.95	33.64	30.78	0.020	0.003	0.008	0.018	0.040	0.051	0.031		1	**0.0004**	**0.0001**	**0.0001**	**0.0013**	0.245
RF16 (*Bacteroidales*)	1.374	1.041	1.607	1.714	2.123	2.154	1.907	0.003	0.004	0.005	0.001	0.006	0.002	0.002		1	1	1	1	1	1
S24-7 (*Bacteroidales*)	2.526	3.670	3.360	3.452	2.955	2.838	2.640	0.001	0.004	0.010	0.007	0.002	0.003	0.008		1	1	1	1	1	1
Unclassified [*Paraprevotellaceae*][d] CF231	0.310	0.719	0.810	0.987	0.970	0.832	1.082	<0.001	0.004	0.002	0.003	0.003	0.002	0.004		1	0.1504	0.058	0.1555	0.7961	0.0868
[*Paraprevotellaceae*] YRC22	0.718	0.834	0.784	0.755	0.708	0.798	0.816	0.002	0.002	0.002	0.001	0.002	0.001	0.001		1	1	1	1	1	1
[*Paraprevotellaceae*] [*Prevotella*]	0.839	0.749	0.924	1.168	1.283	1.528	0.993	0.004	0.002	0.002	0.002	<0.001	0.003	0.003		1	1	1	1	0.3359	1
[*Paraprevotellaceae*] p-2534-18B5	0.095	0.056	0.021	0.048	0.079	0.071	0.049	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
(*Bacteroidales*) SHD-231	<0.001	0.014	0.007	0.008	0.012	0.051	0.155	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002		1	1	1	1	1	1
(*Anaerolinaceae*)	0.322	0.436	0.128	0.048	0.041	0.050	0.047	0.001	0.001	0.001	<0.001	<0.001	<0.001	<0.001		1	0.1175	**0.0003**	**0.0002**	**0.0002**	**0.0001**
YS2 (*Cyanobacteria*)	0.225	0.200	0.077	0.059	0.065	0.008	0.055	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	0.4135	0.0998	0.1218	**0.0001**	0.0682
Fibrobacter	4.892	4.782	7.871	6.430	4.223	5.158	6.889	0.003	0.009	0.010	0.008	0.008	0.015	0.013		1	1	1	1	1	1
Lactobacillus	0.009	0.014	0.037	0.005	1.197	0.039	0.015	<0.001	<0.001	<0.001	<0.001	0.012	<0.001	<0.001		1	1	1	0.4008	1	1
Streptococcus	0.116	0.074	0.138	0.079	1.037	0.077	0.118	<0.001	<0.001	<0.001	<0.001	0.009	<0.001	<0.001		1	1	1	0.7618	1	1

	Mean relative abundance (%)							SEM[a] (*n* = 3[b])							p-value compared to day 1						
	Day							Day							Day						
Day	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Unclassified *Clostridiales*	14.93	15.09	9.75	9.81	8.86	9.49	9.60	0.011	0.018	0.012	0.024	0.018	0.019	0.016	1	**0.0127**	**0.0174**	**0.0028**	**0.0185**	**0.0341**	
Unclassified *Christensenellaceae*	0.692	0.769	0.061	0.053	0.072	0.086	0.084	0.004	0.007	<0.001	<0.001	<0.001	0.001	<0.001	1	**0.0005**	**0.0011**	**0.0132**	**0.0132**	**0.0087**	
Clostridium	0.835	1.082	0.189	0.096	0.151	0.177	0.220	0.003	0.001	<0.001	<0.001	<0.001	<0.001	0.001	1	**0.0006**	**0.0001**	**0.0017**	**0.0059**	**0.016**	
Unclassified *Lachnospiraceae*	10.95	10.74	8.07	5.71	6.81	6.78	7.46	<0.001	0.012	0.004	0.005	0.012	0.014	0.002	1	0.6527	**0.0026**	0.132	0.1651	1	
Anaerostipes	0.416	0.348	0.037	0.022	0.016	0.025	0.032	0.002	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	1	**0.0019**	**0.0008**	**0.0003**	**0.0016**	**0.0016**	
Blautia	0.532	0.396	0.254	0.226	0.227	0.311	0.292	0.001	0.001	<0.001	<0.001	0.001	<0.001	0.001	1	1	0.5559	0.378	1	1	
Butyrivibrio	3.697	4.340	5.445	4.977	5.163	3.805	4.760	0.004	0.018	0.012	0.010	0.011	0.007	0.009	1	1	1	1	1	1	
Coprococcus	0.515	0.486	0.469	0.556	0.560	0.884	0.791	0.002	0.002	0.001	0.002	0.001	0.001	0.002	1	1	1	1	1	1	
Moryella	0.439	0.279	0.303	0.346	0.352	0.310	0.318	0.001	0.001	<0.001	0.001	<0.001	0.002	0.001	1	1	1	1	1	1	
Pseudobutyrivibrio	0.958	1.276	0.940	0.746	0.553	0.578	0.783	0.002	0.002	0.001	0.001	<0.001	0.001	<0.001	1	1	1	0.2451	0.3072	1	
Unclassified *Ruminococcaceae*	5.322	5.684	2.024	1.925	2.127	2.215	2.404	0.003	0.008	0.004	0.002	0.001	0.004	0.004	1	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	
Ruminococcus	6.133	5.085	1.839	1.699	1.737	3.147	2.368	0.010	0.005	0.001	0.004	0.005	0.015	0.008	1	**0.0006**	**0.002**	**0.0049**	0.5677	0.1171	
Unclassified *Veillonellaceae*	0.874	0.406	0.540	0.491	0.618	0.648	0.872	0.005	0.003	0.002	0.001	0.002	0.002	0.004	1	1	1	1	1	1	
Selenomonas	0.156	0.271	0.425	0.697	1.048	0.757	0.859	<0.001	<0.001	0.001	0.001	0.002	0.002	0.001	1	0.7486	0.117	**0.0001**	**0.0053**	**0.0011**	
Succiniclasticum	1.005	0.943	1.963	2.000	2.184	2.157	2.155	0.002	0.003	0.005	0.004	0.004	0.003	0.002	1	**0.0077**	**0.0119**	**0.0038**	**0.0045**	**0.0042**	
Unclassified [*Mogibacteriaceae*]	0.733	0.618	0.533	0.558	0.672	0.634	0.753	0.001	<0.001	0.001	0.001	0.002	0.002	0.003	1	1	1	1	1	1	
Mogibacterium	0.886	0.963	0.710	0.459	0.670	0.534	0.605	<0.001	0.001	0.002	0.001	0.002	0.002	0.001	1	1	0.0919	1	0.653	1	
Bulleidia	0.241	0.376	0.243	0.311	0.442	0.338	0.466	<0.001	0.001	0.001	0.001	0.001	<0.001	0.001	1	1	1	1	1	1	
RFN20 *Erysipelotrichaceae*	0.589	0.650	0.255	0.214	0.273	0.151	0.160	0.001	0.003	0.001	0.001	0.001	<0.001	<0.001	1	1	0.3344	1	0.0653	0.1033	
Unclassified *Alphaproteobacteria*	0.088	0.014	0.029	0.019	0.046	0.042	0.018	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Unclassified *Rickettsiales*	0.030	0.005	0.013	0.034	0.016	0.040	0.029	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Unclassified *Succinivibrionaceae*	0.099	0.015	0.123	0.336	0.263	0.157	0.167	0.001	<0.001	<0.001	0.001	0.002	0.001	0.001	1	1	1	1	1	1	
Treponema	1.084	1.766	3.720	3.997	2.654	2.984	3.221	0.001	0.008	0.007	0.008	0.004	0.005	0.003	1	**0.0002**	**0.0001**	0.0724	**0.0148**	**0.0038**	
TG5 (*Dethiosulfovibrionaceae*)	0.009	0.032	0.023	0.033	0.040	0.013	0.014	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
F16 (TM7)	0.103	0.080	0.013	0.040	0.087	0.107	0.064	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	
Unclassified *Anaeroplasmataceae*	0.028	0.025	0.032	0.057	0.020	0.025	0.010	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1	1	1	1	1	1	

	Mean relative abundance (%)							SEM[a] (*n* = 3[b])							p-value compared to day 1						
	Day							Day							Day						
Day	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**1**	**3**	**4**	**5**	**6**	**7**	**8**
Anaeroplasma	0.216	0.430	0.199	0.179	0.195	0.165	0.125	0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001		1	1	1	1	1	1
RF39 (*Mollicutes*)	0.478	0.351	0.243	0.352	0.573	0.673	0.682	0.001	0.001	0.001	0.001	0.001	0.002	0.002		1	1	1	1	1	1
Others[e]	2.257	2.182	1.477	1.536	1.686	1.453	1.738	0.003	0.003	0.002	0.001	0.002	0.002	0.002		1	0.1418	0.2962	1	0.0636	1

[a]Standard error of the mean.

[b]No. of sheep.

[c]Indicates the group contains sequences not classified down to the genus level.

[d]Square brackets indicate suggested but not verified taxon designations;

[e]A small proportion of sequences that were not present at > 0.5% in even one of the samples.

^{13}C-labelled acetate, propionate and butyrate measured over short-term (8 h)

The incorporation of $^{13}CO_2$ into ^{13}C-acetate, ^{13}C-propionate and ^{13}C-butyrate was measured using GC-IRMS (SPME; Chapter 2; Section 2.18.5). The ratios of $^{13}C/^{12}C$ in acetate, propionate and butyrate were determined after 8 h of *in vitro* incubation of rumen fluid pre-treatment (day 1; Table A.3.8) and during the inhibitor treatment period (day 8; Table A.3.9) for all the three groups of sheep (control, acetylene-treated and chloroform-treated). The enrichment data from IRMS combined with the ruminal VFA data (GC-FID) were used to calculate the amount of excess labelled acetate (^{13}A), propionate (^{13}P) and butyrate (^{13}B) present using the equations described in Chapter 4. The data were used to correct the calculations of the amount of acetate formed from homoacetogenesis (A_{ha}) and butyrate formed from homobutyrogenesis (B_{hb}) in the pre-treatment period (day 1; Table A.3.8) and during the treatment period (day 8; Table A.3.9).

Table A.3.8 Mean ratios of $^{13}C/^{12}C$ and amounts of ^{13}A, ^{13}P and ^{13}B produced after 8 h *in vitro* incubation of rumen contents collected in the pre-treatment period (day 1).

Pre-treatment period (day 1): Mean (± SEM[a]) after 8 h of incubation (n = 4 or 3[b])							
Control sheep							
$NaH^{13}CO_3$ not added							
Inhibitors	**rA_{Uferm}**	**rP_{Uferm}**	**rB_{Uferm}**	**$rCO_{2\ Uferm}$**			
None	0.01093 (± 0.00001)	0.01102 (± 0.00001)	0.01088 (± 0.00002)	0.01112 (± 0.00000)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	**rA_{Lferm}**	**rP_{Lferm}**	**rB_{Lferm}**	**$rCO_{2\ Lferm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.01108 (± 0.00002)	0.01522 (± 0.00006)	0.01097 (± 0.00005)	0.02879 (± 0.00033)	0.00018 (± 0.00002)	0.00162 (± 0.00008)	0.00003 (± 0.00001)
BES	0.01124 (± 0.00003)	0.01549 (± 0.00004)	0.01101 (± 0.00007)	0.02907 (± 0.00028)	0.00026 (± 0.00003)	0.00234 (± 0.00010)	0.00004 (± 0.00001)
$CHCl_3$	0.01102 (± 0.00001)	0.01563 (± 0.00013)	0.01098 (± 0.00006)	0.02909 (± 0.00034)	0.00008 (± 0.00001)	0.00257 (± 0.00022)	0.00003 (± 0.00001)
Acetylene-treated sheep (before treatment)							
$NaH^{13}CO_3$ not added							
Inhibitors	**rA_{Uferm}**	**rP_{Uferm}**	**rB_{Uferm}**	**$rCO_{2\ Uferm}$**			
None	0.01094 (± 0.00001)	0.01105 (± 0.00002)	0.01089 (± 0.00002)	0.01112 (± 0.00000)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	**rA_{Lferm}**	**rP_{Lferm}**	**rB_{Lferm}**	**$rCO_{2\ Lferm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.01107 (± 0.00001)	0.01535 (± 0.00011)	0.01098 (± 0.00004)	0.02898 (± 0.00078)	0.00015 (± 0.00002)	0.00185 (± 0.00009)	0.00002 (± 0.00001)

BES	0.01119 (± 0.00003)	0.01568 (± 0.00009)	0.01100 (± 0.00004)	0.02928 (± 0.00061)	0.00021 (± 0.00003)	0.00268 (± 0.00016)	0.00003 (± 0.00001)
$CHCl_3$	0.01101 (± 0.00001)	0.01570 (± 0.00013)	0.01099 (± 0.00004)	0.02975 (± 0.00065)	0.00006 (± 0.00001)	0.00280 (± 0.00017)	0.00002 (± 0.00001)
Chloroform-treated sheep (before treatment)							
$NaH^{13}CO_3$ not added							
Inhibitors	***r*A$_{Uferm}$**	***r*P$_{Uferm}$**	***r*B$_{Uferm}$**	***r*CO$_{2\ Uferm}$**			
None	0.01093 (± 0.00001)	0.01105 (± 0.00004)	0.01087 (± 0.00002)	0.01112 (± 0.00000)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	***r*A$_{Lferm}$**	***r*P$_{Lferm}$**	***r*B$_{Lferm}$**	***r*CO$_{2\ Lferm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.01106 (± 0.00002)	0.01489 (± 0.00012)	0.01094 (± 0.00004)	0.02906 (± 0.00022)	0.00014 (± 0.00002)	0.00130 (± 0.00007)	0.00002 (± 0.00000)
BES	0.01115 (± 0.00003)	0.01534 (± 0.00008)	0.01095 (± 0.00003)	0.02995 (± 0.00031)	0.00016 (± 0.00002)	0.00191 (± 0.00003)	0.00002 (± 0.00000)
$CHCl_3$	0.01101 (± 0.00000)	0.01517 (± 0.00010)	0.01093 (± 0.00002)	0.02966 (± 0.00036)	0.00005 (± 0.00001)	0.00189 (± 0.00005)	0.00002 (± 0.00000)

[a]Standard error of the mean.

[b]No. of sheep (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).

Table A.3.9 Mean of ratios of $^{13}C/^{12}C$ and amounts of ^{13}A, ^{13}P and ^{13}B produced after 8 h *in vitro* incubation of rumen contents collected in the treatment period (day 8).

Treatment period (day 8): Mean (± SEM[a]) after 8 h of incubation (n = 4 or 3[b])							
Control sheep							
$NaH^{13}CO_3$ not added							
Inhibitors	**rA_{Uferm}**	**rP_{Uferm}**	**rB_{Uferm}**	**$rCO_{2\ Uferm}$**			
Assay	0.01092 (± 0.00000)	0.01098 (± 0.00004)	0.01086 (± 0.00000)	0.01113 (± 0.00000)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	**rA_{Lferm}**	**rP_{Lferm}**	**rB_{Lferm}**	**$rCO_{2\ Lferm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.01109 (± 0.00002)	0.01523 (± 0.00017)	0.01094 (± 0.00026)	0.02825 (± 0.00001)	0.00020 (± 0.00003)	0.00176 (± 0.00008)	0.00002 (± 0.00000)
BES	0.01123 (± 0.00004)	0.01540 (± 0.00016)	0.01097 (± 0.00041)	0.02820 (± 0.00002)	0.00024 (± 0.00003)	0.00248 (± 0.00013)	0.00003 (± 0.00000)
$CHCl_3$	0.01102 (± 0.00001)	0.01540 (± 0.00017)	0.01094 (± 0.00038)	0.02843 (± 0.00001)	0.00007 (± 0.00001)	0.00252 (± 0.00013)	0.00002 (± 0.00000)
Acetylene-treated sheep (during treatment)							
$NaH^{13}CO_3$ not added							
Inhibitors	**rA_{Uferm}**	**rP_{Uferm}**	**rB_{Uferm}**	**$rCO_{2\ Uferm}$**			
None	0.01090 (± 0.00001)	0.01094 (± 0.00003)	0.01085 (± 0.00000)	0.01111 (± 0.00001)			
$NaH^{13}CO_3$ added (3 mM)							
Inhibitors	**rA_{Lferm}**	**rP_{Lferm}**	**rB_{Lferm}**	**$rCO_{2\ Lferm}$**	**^{13}A (µmol/bottle)**	**^{13}P (µmol/bottle)**	**^{13}B (µmol/bottle)**
None	0.01169 (± 0.00009)	0.01573 (± 0.00014)	0.01104 (± 0.00043)	0.02929 (± 0.00002)	0.00065 (± 0.00009)	0.00296 (± 0.00014)	0.00005 (± 0.00001)

BES	0.01171 (± 0.00008)	0.01575 (± 0.00013)	0.01104 (± 0.00038)	0.02914 (± 0.00002)	0.00065 (± 0.00008)	0.00293 (± 0.00014)	0.00005 (± 0.00001)
$CHCl_3$	0.01104 (± 0.00002)	0.01564 (± 0.00005)	0.01097 (± 0.00035)	0.02916 (± 0.00001)	0.00010 (± 0.00001)	0.00301 (± 0.00017)	0.00003 (± 0.00000)

Chloroform–treated sheep (during treatment)

$NaH^{13}CO_3$ not added

Inhibitors	rA_{Uferm}	rP_{Uferm}	rB_{Uferm}	$rCO_{2\ Uferm}$
None	0.01091 (± 0.00000)	0.01108 (± 0.00016)	0.01086 (± 0.00000)	0.01111 (± 0.00000)

$NaH^{13}CO_3$ added (3 mM)

Inhibitors	rA_{Lferm}	rP_{Lferm}	rB_{Lferm}	$rCO_{2\ Lferm}$	^{13}A (µmol/bottle)	^{13}P (µmol/bottle)	^{13}B (µmol/bottle)
None	0.01146 (± 0.00004)	0.01535 (± 0.00002)	0.01099 (± 0.00042)	0.02851 (± 0.00001)	0.00047 (± 0.00002)	0.00261 (± 0.00006)	0.00003 (± 0.00000)
BES	0.01148 (± 0.00006)	0.01546 (± 0.00006)	0.01099 (± 0.00008)	0.02885 (± 0.00002)	0.00049 (± 0.00003)	0.00268 (± 0.00006)	0.00003 (± 0.00000)
$CHCl_3$	0.01102 (± 0.00002)	0.01528 (± 0.00011)	0.01094 (± 0.00030)	0.02848 (± 0.00001)	0.00009 (± 0.00001)	0.00267 (± 0.00004)	0.00002 (± 0.00000)

[a]Standard error of the mean.

[b]No. of sheep (control and acetylene-treated sheep, $n = 4$; chloroform-treated sheep, $n = 3$).