finalreport

Project code: B.CCH.1018

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Date published: December 2011

PUBLISHED BY

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Microbial ecology of hydrogenotrophic rumen microorganisms in response to methane inhibitors

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Management of hydrogen in the rumen is an important factor to be considered when developing strategies to control ruminant methane emissions since hydrogen can impair digestion and fermentation if it accumulates. The aims of this project were to analyse the effect of a methane inhibitor (bromochloromethane) on methane production, metabolic H flux and subsequent responses in SCFA production and rumen microbial community in small ruminants. It was hypothesized that the rumen microbiota would adapt to inhibition of methanogenesis and shift fermentation to reductive processes which would consume more reducing equivalents, but excessive H_2 gas would still accumulate and impair fibre digestion. As predicted, the methane-inhibited rumen appeared to adapt to the high H_2 levels by shifting fermentation to propionate which was mediated by an increase in the population of hydrogen-consuming *Prevotella, Selenomonas and Porphyromonas* spp. As the rumen adapted to the high H_2 concentration the flow of metabolic hydrogen into SCFA increased by >20% but the majority of 2H (>80%) which is normally consumed in methane formation was expelled by the animal. Therefore consumption of this excess hydrogen into yielding substrates for the animal will require the provision of dietary supplements to drive hydrogen uptake or augmentation of minor hydrogenotrophic pathways such as autotrophic reductive acetogenesis.

Executive Summary

Enteric fermentation in livestock accounted for 11.5% of Australia's net total greenhouse gas emissions in the year 2009 (Australian Greenhouse Office, 2011). In rumen fermentation, several pathways involving both hydrogen-producing and -consuming steps are involved in the conversion of feedstuffs into various fermentation end products such as SCFA (Ungerfeld & Kohn 2006, Moss *et al* 2000). Although metabolic H in the rumen is incorporated in fermentation end products by bacteria, methanogenic archaea (methanogens) consume the greater majority of metabolic H to obtain energy for their metabolism and finally release methane, which accounts for 2-12% loss of the metabolic energy from feed (Johnson & Johnson 1995, Moss *et al* 2000, Morgavi *et al* 2010). Therefore management of metabolic H and methane production in the rumen is an important factor to be considered, when developing strategies to reduce greenhouse gas emissions and improve efficiency of energy utilization from feed.

It is known that many chemical agents such as ionophores (e.g. monensin), unsaturated fatty acids, sulfate, nitrate, fumarate, and halogenated methane analogues (e.g. bromochloromethane (BCM)) are able to reduce methane production from ruminants (Johnson & Johnson 1995, Morgavi *et al* 2010, McSweeney & McCrabb 2002, Itabashi 2002). BCM complexed in cyclodextrin (CD) (BCM-CD) results in the sustained inhibition of methane production when fed to ruminants (May *et al* 1995, McCrabb *et al* 1997, Tomkins & Hunter 2004). It is predicted that H_2 gas would then accumulate in the rumen with the suppression of growth of ruminal methanogens, leading to a decrease in microbial rumen function (Janssen 2010).

The aims of this study were to establish a small ruminant model and analyse the effect of BCM-CD on *in vivo* methane production, metabolic H flux and subsequent responses in SCFA production and rumen microbial community structure of goats. Varying dose levels of BCM-CD were administered to the rumen of goats fed a 1.1 X maintenance diet of roughage and concentrate (1:1) in open-circuit respiration chambers. It was hypothesized that (1) the rumen microbiota would adapt to inhibition of methanogenesis and shift fermentation to reductive processes, which would consume more reducing equivalents, but (2) excessive H_2 gas would still accumulate and impair fibre digestion.

Through the use of modern molecular based techniques such as quantitative PCR to assess the contribution of specific bacterial groups to the environment, along with microbial ecology techniques to monitor entire microbial population shifts from varying levels of BCM administration, metagenomics to identify the functional potential found within the bacteria and metatranscriptomics to validate the activation of bacterial fermentation pathways it will be possible to assess the effect BCM on the rumen.

Results from this study showed that a dose-dependent inhibitory effect of BCM on the goat rumen was observed for methane production. Doses of net BCM, 0.04, 0.16, and 0.4 g/100 kg LW, reduced methane production by 5, 71, 91%, respectively, compared to controls and there was no effect on maintenance feed intake and NDF digestibility. This indicates fibre digestion was not compromised on a highly digestible diet even though H_2 concentration increased markedly in the rumen due to inhibition of methanogenesis. It is unlikely that BCM would be used commercially for methane inhibition even though it shows an intensive effect on methane reduction, because this compound is regarded itself as a greenhouse gas (Solomon *et al* 2007). However, its does allow us to manipulate the rumen and assess the microbial and animals response to the increased rumen hydrogen concentrations. In the first instance it is apparent that the rumen microbiology does not collapse with

detrimental flow on effects to the animal host. Rumen fermentation end products were mostly unchanged with respect to the addition of BCM, but significant increases in propionate and iso-valerate were detected at the mid and high doses. With more hydrogen being available reductive processes involving propionate production and reductive acetogenesis become thermodynamically favorable (Ungerfeld & Kohn 2006). However, there was no change to acetate levels, which possibly indicates that reductive acetogenic bacteria have not contributed significantly to the consumption of the accumulated H_2. On the other hand the acetate concentration, could reflect a decreased production of acetate by oxidative pathways combined with increased production from reductive acetogenesis. No measures were made that would allow this to be ascertained from this trial, but future studies with the use of labeled substrate required for autotrophic growth of reductive acetogens would resolve this issue. The consistent increase in branched chain fatty acids as an adaptive response of the rumen microbiota to BCM and high H may have resulted from proteolytic activity associated with the greater abundance of *Prevotella* species.

Even though the flow of 2H into SCFA increased by >20% at the high BCM dose, it was observed and calculated that the majority of 2H available from reduced methane formation flowed into H_2 gas instead of SCFA and this still represents an energy loss to the animal.

This study has provided the first analysis *in-vivo* of the relationship between total methanogen numbers and level of methane formation from a normal rumen to nearly zero methanogenesis. The methanogen population decreased by 5 and 70 fold at the mid and high BCM doses compared with the controls which resulted in a 71 and 91 % reduction respectively in methane production. Surprisingly a half log reduction in the normal methanogen population correlated with >50% reduction in methane. This probably indicates that the relative methanogenic activity of different archaeal species in the rumen plays a greater role in determining methane output than the absolute number of methanogens.

The population of the highly cellulolytic bacterium *F. succinogenes* increased slightly in relation to BCM treatment. Unlike the cellulolytic *Ruminococci* and fungal species in the rumen, *F. succinogenes* does not produce H_2 and is not susceptible to H_2 accumulation. Other fibrolytic bacteria and anaerobic rumen fungi rely on interspecies H_2 transfer for H_2 utilization and are therefore affected by the loss of the methanogens from this system with concomitant reductions in fiber digestion. The protozoal population did not appear to be affected by BCM even though ecto- and endo-symbiotic methanogens associated with protozoa would have been inhibited.

In depth microbial ecology and metagenomic studies allowed for the first time a high-resolution observation into the changes in rumen microbial populations with respect to abundance and presumed level of metabolic activity within the system. By targeting the microbial DNA it is possible to estimate a microbial members contribution to the rumen system while RNA will produce an estimate of its contributed metabolic activity to the rumen. The results from this study show that populations that would normally be considered as minor contributors to rumen function are highly active and are likely to elicit the major responses to higher hydrogen concentrations in the absence of methanogens. Bacteria representing *Fibrobacter, Porphyromonad, Prevotella, Selenomonas* and *Treponema* species were not only found to be more active but were also shown to be intricately associated with each other producing a coordinated response to the higher hydrogen concentrations.

Metagenomic analysis was dominated by genomic content from *Prevotella* species and showed their potential for transforming succinate through to propionate. However, metatranscriptomics analysis revealed that while some activity could be associated with the *Prevotella* species, *Porphyronad* and *Selenomonas* species also dominated the pathways that resulted in the production of propionate at the

reduction of hydrogen and that these species are likely to better targets for promoting changes of this nature within the rumen.

In summary, this project has identified the hydrogenotrophic bacteria in the digestive tract of ruminants that respond to excess hydrogen and which should be stimulated in strategies to reduce methane emissions. The rumen microbial ecosystem compensates for the increases in hydrogen without any detrimental effects on fibre digestion and animal production on a high quality diet but responses on roughage diets typical of tropical production systems need to be tested. Consumption of this excess hydrogen into yielding substrates for the animal will require the provision of dietary supplements to drive hydrogen uptake or augmentation of minor hydrogenotrophic pathways such as autotrophic reductive acetogenesis. With this knowledge more informed models can be developed which will help the researcher and producer to better understand and manage changes within the rumen as they relate to different production systems.

Contents

1 Background - Section

1.1 Purpose and description

Methane produced through enteric fermentation in livestock accounted for 11.5% of Australia's net total greenhouse gas emissions in the year 2009 (Australian Greenhouse Office, 2011). In rumen fermentation, several pathways involving both hydrogen-producing and consuming steps are involved in the conversion of feedstuffs into various fermentation end products such as SCFA (Ungerfeld & Kon 2006, Moss *et al* 2000). Although metabolic H in the rumen is incorporated in fermentation end products by bacteria, methanogenic archaea (methanogens) consume the greater majority of metabolic H to obtain energy for their metabolism and finally release methane, which accounts for a 2-12% loss of the metabolic energy from feed (Johnson & Johnson 1995, Moss *et al* 2000, Morgavi *et al* 2010). Therefore management of metabolic H and methane production in the rumen is an important factor to be considered, when developing strategies to reduce greenhouse gas emissions and improve efficiency of energy utilization from feed.
It is known that many chemical agents such as ionophores (e.g. monensin), unsaturated fatty acids, sulfate, nitrate, fumarate, and halogenated methane analogues (e.g. bromochloromethane (BCM)) are able to reduce methane production from ruminants (Johnson & Johnson 1995, Morgavi *et al* 2010, McSweeney & McCrabb 2002, Itabashi 2002). BCM is one of the most effective inhibitors, and apparently reduces methane production by interfering with the cobamide-dependent methyl transferase step of methanogenesis (Wood *et al* 1968, Chalupa 1977). BCM complexed in cyclodextrin (CD) (BCM-CD) results in the sustained inhibition of methane production when fed to ruminants (May *et al* 1995, McCrabb *et al* 1997, Tomkins & Hunter 2004). It is predicted that H_2 gas would then accumulate in the rumen with the suppression of growth of ruminal methanogens, leading to a decrease in microbial rumen function (Janssen 2010).

Ungerfeld and Kohn (2006) provided an excellent overview of the thermodynamics of ruminal fermentation and identified several strategies for utilising hydrogen in the rumen as an alternative to methanogenesis. Fumarate and malate have been used by several investigators to stimulate succinate/propionate producers in the rumen which compete with methanogens for hydrogen. Many of these organisms use the succinate-propionate (randomizing) pathway as a major route for propionate synthesis in the rumen (Baldwin et al. 1963). In this pathway, malate is dehydrated to fumarate, and the reduction of fumarate to succinate is coupled to ATP synthesis. Succinate is either an intermediate or an end-product in the pathway of different rumen bacteria. However, several researchers have shown in mixed ruminal cultures that fumarate (and malate) is converted to propionate and acetate in varying proportions (Ungerfield & Kohn 2006; Ungerfield *et al*. 2007). The relative amounts of propionate and acetate formed from fumarate will impact on the hydrogen pool available to methanogens. Stoichiometrically, propionate production from fumarate consumes one pair of reducing equivalent while acetate production from fumarate releases two pairs of reducing equivalents. Therefore the production of acetate from fumarate is counterproductive when the objective is to reduce hydrogen available for methane production. It is important therefore to identify the microorganisms involved in these pathways and determine the physiological and biochemical conditions which favour propionate rather than acetate production from fumarate.

This project will provide this understanding by identifying and characterising the microbiology and genetics underpinning the alternative hydrogenotrophic pathways in ruminants, using metagenomics. The knowledge and resources arising from this project can then be used as the basis of an integrated approach to develop mechanisms that successfully inhibit rumen methanogens and (or) methane emissions from ruminant livestock.

2 Project Objectives

1. Establish a small ruminant model with and without the methane inhibitor (bromochloromethane) to promote metabolically active pathways in the rumen that shift from methanogenesis tomalternative pathways of hydgrogen consumption.
2. Quantify the effects of reduced methanogenesis on rumen fermentation and digestion.
3. Through the use of real time PCR assays and microarray analysis, quantify the effects of reduced methanogenesis on microorganisms that are known to be involved in fibre digestion and alternative hydrogen utilisation.
4. Through the use of metagenomic techniques determine the microorganisms and genes involved in hydrogenotrophy in response to reduced methanogenesis and higher concentrations of hydrogen.
5. Isolate microbes that are responsible for the alternative routes of hydrogenotrophy in the rumen.
6. Based on the outputs of the previous objectives, conduct an animal supplementation trial that demonstrates the optimal level of methane reduction in the rumen and response in alternative hydrogenotrophy to prevent detrimental effects on rumen fermentation.

3 Methodology

3.1 Animals and experimental design

Three fistulated Japanese native goats (Capra aegagrus hircus, female) 35.7 ± 4.85 kg were fed a 1.1 X maintenance diet of 50% of Timothy hay (*Phleum pratense*) (g/kg; OM 949, NDF 735, CP 49, ash 51) and 50% of concentrate (designed by National Institute of Livestock and Grassland Science (NILGS): corn powder, 30%; barley powder, 25%; wheat bran, 9%; defatted rice bran, 7%; soybean meal, 11.7%; molasses, 3.5%; alfalfa meal, 7%; beet pulp, 5%; Calcium carbonate, 1.2%; sodium chloride, 0.5%; vitamin premixed, 0.1%) (g/kg; OM 937, NDF 243, CP 185, ash 63), with which CD (0.46 g/100 kg LW) was supplemented, (376.5 ± 39.0 g each) for an adaption period of 14 days prior to gas measurements and sampling. A sampling period of eight days followed and served as the control collection period for all animals. Within the sampling period goats were placed into the respiration chambers for a period of three days for analysis of rumen gas production. Animals were then adapted to a low dose of BCM-CD (0.5 g/100 kg LW), which was premixed in the maintenance diet without CD, for 8 days prior to sampling in the respiration chambers for three days. Doses were then increased to a mid dose (2 g/100 kg LW) and after 8 days of adaption, there was a further three days of sampling. A final high dose (5 g/100 kg LW) of BCM was administered and a similar adaption and sampling regime was followed as before, with gas analysis performed in the respiration chambers. The BCM-CD containing 8% BCM was prepared by Ensuiko Sugar Refining Co. Ltd. (Yokohama, Japan). Rumen samples were collected at the end of each respiration-sampling period (3 days) for analysis of fermentation end-products and microbial ecology. Digestibility measurements and measurement of methane production in respiration chambers were performed according to the techniques and procedures described by Bhatta (2008). Methane concentration was accurately measured by sampling gas from the outlet of a chamber. Hydrogen concentrations in a chamber were measured by a hydrogen monitor (Custom-made device, TAIYO Instruments Inc., Osaka, Japan), which was installed in each chamber. The animal experiments were carried out in accordance with a protocol approved by the Guide for the Care and Use of Experimental Animals (Animal Care Committee, NILGS).

3.2 Organic acid sampling and analysis

For the detection of SCFA, a 1ml-sample of rumen fluid was taken at various time points for analysis. After samples were centrifuged (12 000g, 10 min), 600 µl of sample was combined with 150 µl of meta-phosphoric/internal standard solution (20% meta-phosphoric acid/0.24% 4-methyl valeric acid). Organic acids analysis was performed using a Shimadzu GC-17A gas chromatograph on a packed glass column (2 m length; 0.6 mm OD, 0.2 mm ID) containing 10% FFAP/1% H_3PO_4 on Chromasorb WAW 100/120 mesh. The C2–C5 acids were separated over 16 min using nitrogen as a carrier at 12 ml/min. Peaks were detected by a flame ionization detector (Playne 1985). Lactate concentrations were determined from the supernatants of the centrifuged samples with the D-lactate and L-lactate assay kit (K-DLATE, Megazyme International Ireland Ltd., Wicklow, Ireland) according to the manufacturer's instructions.

3.3 Calculation of hydrogen balance

The estimated recovery of metabolic hydrogen in the form of reduced protons (H) can be calculated from 2H utilised (2HU) and 2H produced (2HP) in rumen fermentation. The 2HP as a fermentation intermediate and 2H utilised in SCFA and methane were estimated from the amount and molar proportions of acetate (C2), propionate (C3), butyrate (C4), isovalerate (Ci5) and valerate (C5) and molar concentration of methane as described in the following (Goel *et al* 2009, Deyemer 1991) equations:

2H produced (2HP) (mmol/l) = 2 x C2 + C3 + 4 x C4 + 2 x Ci5 + 2 x C5,
2H utilised in SCFA (2HUS) (mmol/l) = 2 x C3 + 2 x C4 + C5,
2H recovery in SCFA (%) = (2HUS/2HP) x 100

Since administration of BCM induced a marked increase in H_2 gas production, which reached unmeasurable level (>65 ppm, Fig. 1), it was predicted that 2HU comprised 2HUS, 2H recovery in methane (2HUM), and 2H recovery in H2 (2HUH). Therefore, 2HU was calculated as

2HU = 0.9 x 2HP = 2HUS + 2HUM + 2HUH (eq. 1)

because the recovery rate of metabolic hydrogen, which is 2HU/2HP, is estimated as 0.9 (Moss *et al* 2000, Deyemer 1991, Faicheny *et al* 1999), however in the controls there was minimal H_2 production since methane formation was not inhibited, and thus 2HU was calculated according to Goel *et al*. (2009) as:

2HU = 2HUS + 2HUM = (2 x C3 + 2 x C4 + C5) + (4 x methane) (eq.2)

Therefore the equation used to calculate methane production in controls (Mcont) was: Mcont = (2HU - 2HUS)/4 = [(0.9 x 2HP) - 2HUS]/4. Methane production at the low, mid and high doses of BCM (Mbcm) (mmol/l) was calculated by using Mcont and methane reduction rates (MR (%)), which were calculated from methane production in respiration chambers (Mrc) (mol/head/day), as: Mbcm = Mcont x (100 - MR)/100. H_2 production at each dose of BCM (Hbcm) was calculated as a modification of eq. 1 and eq. 2:

Hbcm (mmol/l) = 2HU- (2HUS + 2HUM) 2HU- [(2 x C3 + 2 x C4 + C5) + (4 x Mbcm)].

The amount of H_2 production in respiration chambers (Hrc) was estimated in mol as Hrc (mol/head/day) = Mrc (mol/head/day) x Hbcm/Mbcm. 2H utilised in H2 (2HUH) was estimated as: 2HUH (%) = (Hbcm/2HP) x 100.
The relationship between measured methane production in chambers and (C2 + C4)/C3 ratio were plotted on a chart according to the method reported by Moss *et al.* (2000), in order to obtain an approximate curve.

3.4 Monitoring microbial populations

3.4.1 DNA extractions

DNA extractions were carried out on rumen samples collected from the goats using the cetyltrimethylammonium bromide (CTAB) method of Brookman et al. (2005) with minor modifications as follows: samples were centrifuged (13,000 X *g* for 5 min), and the supernatant was removed before DNA extraction. Cells were homogenized with 200 mg of silica-zirconium beads (1:1 mixture of 0.1- and 1.0-mm beads; Biospec, Bartlesville, OK) and 800 µl of CTAB buffer in a Mini-Beadbeater-8 (Biospec) on maximum speed for 2 min, twice. Samples were incubated at 70°C for 20 min and centrifuged at 10,000 X *g* for 10 min, and the supernatant was mixed with 500 µl of 25:24:1 phenol-chloroform-isoamyl alcohol (Fluka BioChemika, Buchs, Switzerland).

3.4.2 Quantitative PCR

Quantitative PCR (qPCR) for monitoring three cellulolytic rumen bacterial species; *Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens*, anaerobic rumen fungi and methanogen populations were performed using published primers and assay conditions (Denman & McSweeney 2006, Denman *et al* 2007,Kang *et al* 2010). Quantitative PCR Primers were designed to target specific operational taxonomic units (OTUs) identified from phylogenetic studies (see below). Primers were designed from multiple alignments of DNA sequences of relevant target sequences and closely related full length sequences. Primers were then compared with sequences available at the National Centre for Biotechnology Information (NCBI) via a Basic Local Alignment Search Tool (BLAST) search to ascertain primer specificity (Altschul *et al*. 1990). In addition the primers were used to amplify products from rumen samples and clone libraries of these fragments were sequenced and compared for their specificity.

Changes in targeted populations were calculated using a relative quantification calculation and the $2^{-\Delta\Delta CT}$ method with the control period used as the calibrator and total bacterial ct values used as the reference value (Livak & Schmittgen 2001).
Quantitative PCR (qPCR) assays were performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). Assays were set up using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Optimisation of assay conditions was performed for primer, template DNA and $MgCl_2$ concentrations. An optimal primer concentration of 300 nM, a final $MgCl_2$ concentration of 3 mM and DNA template concentration of between 1 and 100ng were used for each assay under the following cycle conditions: one cycle of 50°C for 2 min and 95°C for 2 min for initial denaturation, 40 cycles at 95°C for 15 s and 56°C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each denaturation and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by raising the temperature at a rate of 1°C/30 sec from 60°C to 95°C. Total microbial rumen DNA from sampling time points was diluted 1:10 prior to use in quantitative real time PCR.

3.4.3 Functional gene analysis

Functional gene libraries were constructed from DNA samples collected from the control and high BCM dosing periods. One, targeting methanogenesis using the methyl coenzyme A reductase (mcrA)

(using mcrA forward primer 5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC and mcrA reverse primer 5'-TTCATTGCRTAGTTWGGRTAGTT (Luton *et al.,* 2002)). Two libraries targeting reductive acetogenesis genes: the formyl tetrahydrofolate synthase (FTHFS) (using the FTHFS forward primer 5'-TTYACWGGHGAYTTCCATGC-3'; and FTFHS reverse 5'-GTATTGDGTYTTRGCCATACA-3' (Leaphart & Lovell 2001) and acetyl CoA synthetase (ACS) (using the ACS forward primer 5'-CTBTGYGGDGCIGTIWSMTGG and ACS reverse 5'-AARCAWCCRCADGADGTCATIGG (Gagen *et al* 2010). Amplified PCR products were gel extracted from a 1.5% agarose gel and cloned using the pGEM-Teasy vector system (Promega). Transformed *E.coli* cells harbouring the cloned products were selected and sequenced using BigDye sequencing reagents (ABI). Sequence data was analysed in the ARB software environment (Ludwig *et al.* 2004) and OTU analysis performed using MOTHUR (Schloss *et al.* 2009).

3.5 Metageneomic analysis

3.5.1 16S rDNA analysis

Using high throughput sequencing platforms and barcode "pyrotagging", phylogenetic based methods targeting the 16S rDNA gene were used to deeply characterise the microbial populations present in the rumen for the control and treatment periods. 16S rRNA gene pyrotagging was performed using modified universal bacterial primers (27f and 515r). Specific sequences matching the Roche 454 sequencing adaptor B were added to the 27f primer, while adaptor A was added to the 515r. In addition between the adaptor A sequence and the 16S 515r sequence a 10 bp barcode was inserted. Each individual DNA sample was amplified using the 27f primer and a uniquely barcoded 515r primer. After amplification products were visualised by performing gel electrophoresis. Product quantities were calculated and an equal molar amount of each product was pooled. The pooled products were run in a 1.5 % agarose gel and the product gel extracted and purified prior to submission for 454 pyrosequencing.
Short read sequence data generated using 454 sequencing was analysed using the QIIME: Quantitative Insights Into Microbial Ecology software package (Caporaso *et al* 2010), MOTHUR (Schloss *et al.* 2009) for generation of OTU clusters, alpha and beta diversity measures and distance calculations and R with the corpcor, qgraph and sna packages for calculation of interaction networks that were then visualised using cytoscape (Smoot *et al.* 2011).

3.5.2 Meta-genomic analysis

A metagenomic assessment of the goat microbiome from the control and high BCM dosing using 454 pyro-sequencing was undertaken. High molecular weight DNA extracted from the three goat samples were pooled together and nebulized and 454 adapter fragments were added. The DNA was then amplified on small DNA-capture beads in a water-in-oil emulsion. Each DNA-bound bead was placed into a ~44 μm well on a PicoTiterPlate for massively-parallel pyrosequencing by 454 GS-Flx using titanium chemistry (Tringe and Hugenhotlz 2008). One half plate of sequencing was performed on each library, data generated from the 454 sequencing run was passed through a quality filtering pipeline which initially involves de-replication of the data which occurs at the emulsion PCR step where multiple reads from a single template occur due to amplified DNA attaching to empty beads. Data was then analysed for the occurrence of ribosomal DNA reads using hidden Markov models implemented in the software package hmm_rRNA (Huang *et al.* 2009). Assembly of metageneomic

sequences into larger contiguous sequences (contigs) was implemented with Newbler (Roche ver. 2.6). Contigs and orphaned reads (unassembled sequences) were annotated using the MG-RAST server, involving phylogenetic placement and functional gene annotation (Meyer *et al.* 2008).

3.5.3 Meta-transcriptomic analysis

Goat rumen samples collected throughout the trial were used for the extraction of total microbial RNA. Samples were placed immediately into and equal volume of RNAlater (Invitrogen) to preserve RNA transcripts prior to extraction using an RNeasy purification kit (QIagen). Quality of extracted RNA was visualized on a bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent), before preparation of material for RNA sequencing. Samples from the control period and High BCM treatment were pooled separately and paired end RNAseq libraries were created and run on a lane each on an Illumina Hiseq 2000 instrument (Illumina).
RNAseq data was first passed through the quality filtering program nesoni clip (Paul Harrison VBC, Monash University) which filters reads on a number of criteria: if the majority of a read is a homopolymer it is filtered out, then clipping out of adapter sequences, clipping of ambiguous bases and removal of bases under a quality threshold (a sanger qual score of 10) before passing a length filter of greater than 50 bp. Quality trimmed sequences are then outputed for paired ends that both pass quality filtering and singles were only one of the paired reads was successful. Ribosomal genes were identified and removed from within the quality paired end reads using hidden Markov models implemented in the software package hmm_rRNA (Huang *et al.* 2009). Remaining non-ribosomal sequences were then subjected to two independent RNAseq analysis methods: 1) mapping of reads to the goat metageneomic data set or relevant microbial genomes downloaded from NCBI using the Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009), 2) *de-novo* assembly of reads into contigs was performed using the software packages Velvet (Zerbino and Birney 2008) and Oases (Zarbino and Schulz 2011) using various kmer values. Differential expressed genes were then identified using R with the edgeR analysis package and annotated using BLAST, PFAM and KEGG databases.

4 Results and Discussion

4.1 Digestibility, rumen gas and organic acid analysis

4.1.1 Digestibility

All feed offered (1.1 X maintenance diet) was consumed by each goat and digestibility data was analysed for the control and highest dose of BCM. BCM did not exhibit a negative effect on digestibility compared to the control period (Table 1).

Table 1. Feed digestibility in goats

	Control*	BCM (high)
DM (%)	68.3 ± 1.49	70.9 ± 2.05
ODM (%)	69.2 ± 1.47	71.8 ± 1.95
NDF (%)	54.7 ± 1.75	59.1 ± 2.62
Energy (%)	65.9 ± 1.71	68.1 ± 2.22

ODM, organic DM; NDF, neutral-detergent fibre.
*Mean values for three animals ± SEM.

4.1.2 Methane and H_2 gas production

Daily quantities of methane produced from animals were reduced with respect to the dose level of BCM (Table 2). Significant decreases in methane production were evident at the mid and high doses with a 71.3% and 91% decrease, respectively, compared to the control. Real-time monitoring of the H_2 gas showed that in the control period levels in expired gases were negligible (Fig.1). When the highest dose of BCM was administered the concentration of H_2 rapidly accumulated in the chamber (above measurable levels >65 ppm) and only decreased in concentration in the latter stages of monitoring for each day in relation to feeding (Fig.1). The estimated H_2 production in respiration chambers (Hrc) is shown in Table 2. Zero H_2 production during the control period was used when estimating H_2 production at various levels of BCM. A low level of H_2 production (544.6 mmol/head/day) was estimated for the low BCM dose (5% methane reduction, MR) compared with H_2 production which significantly increased by 6.4-fold at the high BCM dose (91% MR).

Table 2. Measured CH_4 and estimated H_2 gas production levels in goats at various levels of BCM

	Control[#]	Low	Mid	High
Methane production				
mmol/head/day	949.6 ± 88.70[a*]	902.1 ± 72.58[a]	273.1 ± 84.61[b]	86.0 ± 35.61[b]
mmol/Kg of DMI	1412.4	NA	NA	127.3

mmol/Kg of ODI	1497.7	NA	NA	135
mmol/Kg of NDF	2872.8	NA	NA	260.6
MR (%)		5	71.3	91
H_2 gas production				
mmol/head/day	$0^{a‡}$	544.6 ± 261.9^{ab}	2941.4 ± 1141.2^{bc}	3495.5 ± 237.6^{c}

NA, data not available; DMI, DM intake; ODI, organic dry matter intake; MR, methane reduction on a head basis. # Mean values for three animals ± SEM. * Means in the same row that do not share common superscripts are significantly different (P<0.05). ‡ No hydrogen production is presupposed at control. Hydrogen levels at the low, mid and high doses of BCM were stoichiometrically estimated from methane production, MR, and SCFA concentrations (see text).

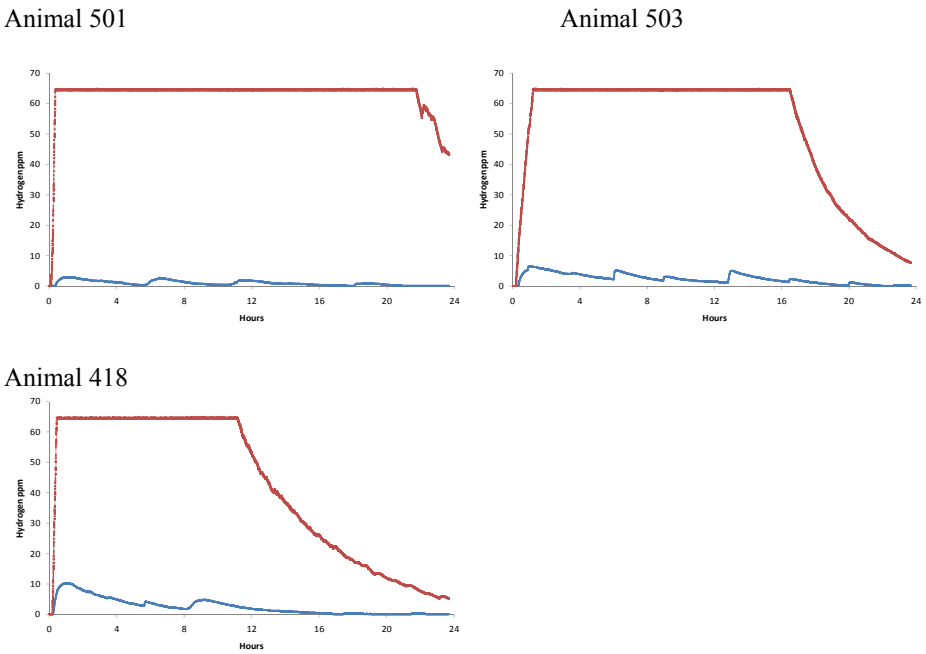


Figure 1. Hydrogen levels (ppm) detected within the rumen for the three individual animals at control (blue) and high dose of BCM (red).

4.1.3 Organic acid production

The total SCFA content ranged from 47.5 to 55.3 mmol/l with no significant difference between treatments. Although concentrations of propionate and iso-valerate were significantly increased in the

high dose (P<0.05), there was no significant change in those of acetate, iso-butyrate, butyrate and valerate (Table 3). Significant decreases of the acetate:propionate ratio was observed at mid and high doses (P<0.05) (Table 3). The lactate concentrations showed no significant difference among treatments (Table 3). However two animals showed a gradual decline in lactate as they transitioned from control to high BCM (data not shown).

Table 3. SCFA and lactate concentrations (mmol/l) in goats at varying levels of BCM

	Control[#]	Low	Mid	High
Total SCFA	47.5 ± 7.10	50.6 ± 3.94	53.6 ± 11.95	55.3 ± 4.18
Acetate	31.0 ± 5.31	33.1 ± 2.75	33.7 ± 8.25	32.0 ± 3.51
Propionate	8.13 ± 1.10[a]*	8.27 ± 0.58[a]	11.07 ± 2.23[ab]	12.73 ± 1.03[b]
i-Butyrate	1.47 ± 0.09	1.50 ± 0.12	1.40 ± 0.08	1.67 ± 0.07
Butyrate	5.37 ± 0.72	6.00 ± 0.50	6.27 ± 1.56	6.50 ± 0.53
i-Valerate	1.53 ± 0.07[a]	1.47 ± 0.09[a]	1.67 ± 0.43[a]	2.47 ± 0.13[b]
Valerate	ND	ND	ND	ND
A:P	3.78 ± 0.15[a]	4.01 ± 0.18[a]	3.01 ± 0.19[b]	2.55 ± 0.35[b]
Lactate	8.61 ± 3.25	6.33 ± 2.67	5.70 ± 1.21	6.51 ± 1.36

ND, not detected; A:P, Acetate:Propionate ratio. # Mean values for three animals ± SEM. * Means in the same row that do not share common superscripts are significantly different (P<0.05).

4.1.4 2H balance calculations

The values of 2H recovered as SCFA are shown in Table 4. Although 28.3 to 28.7% of 2HP could be related to SCFA at nil or low BCM dose, more 2HP (32.7 to 36.0%) was converted into SCFA at mid or high dose. The 2H accounted for as SCFA relative to the control was increased to 125% with a high dose of BCM.

Table 4. 2H incorporated into SCFA

	Control	Low BCM	Mid BCM	High BCM
% of 2HP	28.7 ± 1.0[a*]	28.3 ± 1.2[a]	32.7 ± 2.2[ab]	36.0 ± 4.2[b]
% of control	-	98.5 ± 3.0[a]	114.1± 3.9[b]	125.4 ± 11.4[b]

2HP, metabolic hydrogen produced in rumen fermentation. # Mean values for three animals ± SEM. * Means in the same row that do not share common superscripts are significantly different (P<0.05).

The relationship between methane production (l/day) and the (C2 + C4)/C3 ratio is shown in Fig. 2. A highly significant (P<0.01) linear regression equation was derived as follow;

$$y = 9.65x – 25.7 \ (r^2 = 0.698).$$

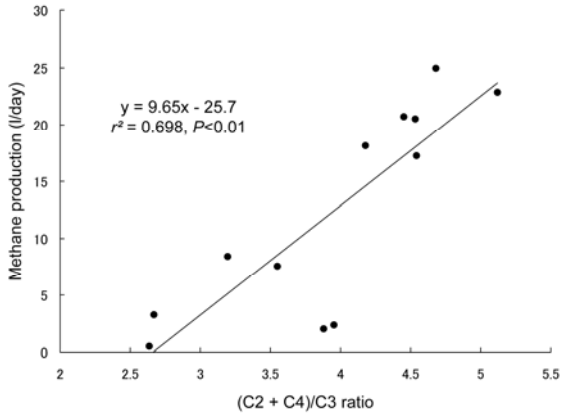


Figure 2. Relationship between methane production (l/day) and (C2 + C4)/C3 ratio. Methane production was measured using a respiration chamber. (C2 + C4)/C3 ratio was determined from concentrations of C2, C3 and C4 in the rumen fluid.

Simple regression analysis revealed the strong correlation ($P<0.01$) between MR level and the percentage of 2HUS, 2HUM, or 2HUH/2HU (Fig. 3). It was estimated that the percentage of 2HUH to 2HU increased from 3.1% at no dose (control) to 55.1% at high dose.

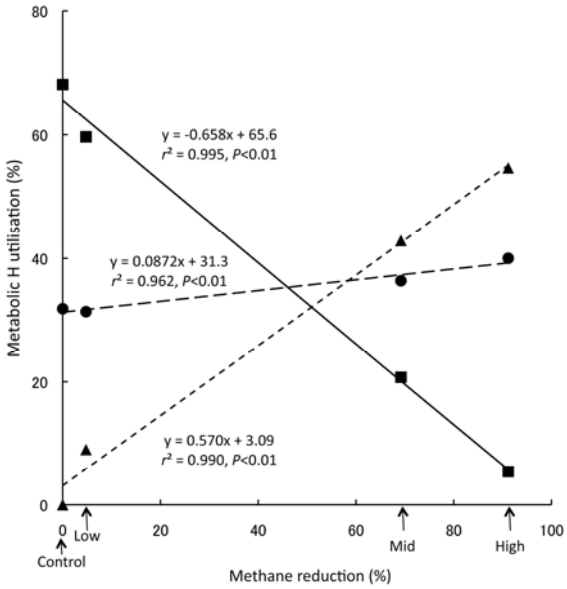


Figure 3. Distribution of metabolic H utilisation (2HU) in SCFA (●), methane (■), or hydrogen (▲) at various level of BCM (Control, Low, Mid and High). Observed values of SCFA concentration and methane production were used for calculations of hydrogen production and 2HU.

The findings show a dose-dependent inhibitory effect of BCM on the goat rumen for methane production. Doses of net BCM, 0.04, 0.16, and 0.4 g/100 kg LW, reduced methane production by 5, 71, 91%, respectively, compared to controls and there was no effect on maintenance feed intake and NDF digestibility. This indicates fibre digestion was not compromised on a highly digestible diet even though H_2 concentration increased markedly in the rumen due to inhibition of methanogenesis. Similarly Goel *et al.* (2009) also showed that BCM did not affect the values for degradability of substrate in an *in vitro* continuous fermentation study when methanogenesis was inhibited. Moreover, Tomkins *et al*. (2009) reported that dry matter intake and average daily gain of beef cattle fed a feedlot diet were not influenced by BCM treatment, in which administration of 0.3g of BCM/100 kg LW reduced almost the same level of methane production as shown at the high dose in this study.
Methane production in goats was significantly reduced at the mid and high BCM doses similar to other *in vivo* and *in vitro* studies. Moreover, the suppression of methane production by BCM led to a large accumulation of H_2, which confirms the theoretical predictions by Janssen (2010). H_2 accumulation in the rumen has also been reported by Trei *et al*. in which methane production in the rumen of sheep or lambs was inhibited by administration of 2,2,2-Trichloroacetamide or hemiacetal of chloral and starch, respectively (Trei *et al* 1971, Trei *et al* 1972). It is unlikely that BCM would be used commercially for methane inhibition even though it shows an intensive effect on methane reduction, because this compound is regarded itself as a greenhouse gas (Solomon *et al* 2007. However, it still remains a goal to further enhance the 2HP flows into SCFA in order to reduce production of greenhouse gases and to increase feed efficiency in ruminants.

Rumen fermentation end products were mostly unchanged with respect to the addition of BCM, but significant increases in propionate and iso-valerate were detected at the mid and high doses. Similar responses have been reported from *in-vivo* and *in-vitro* studies where BCM was used to inhibit methane formation (Goel *et al* 2009, Denman *et al* 2007). Inhibition of methanogenesis in the rumen is usually associated with an increase in propionate, and is believed to be due to the competition for hydrogen (Hungate 1967). With more hydrogen being available reductive processes involving propionate production and reductive acetogenesis become thermodynamically favourable (Ungerfeld & Kohn 2006). There was no change to acetate levels, which possibly indicates that reductive acetogenic bacteria have not contributed significantly to the consumption of the accumulated H_2. The acetate concentration, however, could reflect a decreased production of acetate by oxidative pathways combined with increased production from reductive acetogenesis. This could be resolved in future studies with the use of labelled substrate required for autotrophic growth of reductive acetogens. The consistent increase in branched chain fatty acids as an adaptive response of the rumen microbiota to BCM may have resulted from proteolytic activity associated with the greater abundance of *Prevotella* species.

It was estimated that about 33-36 % of 2HP in the rumen could be recovered as SCFA at mid and high BCM dose, whereas about 28% of 2HP was converted into SCFA at the control and low dose. In the *in vitro* study of Goel *et al*. (2009), the addition of BCM (10 μM) led to a 94% reduction of methane production and increased the 2H recovered as SCFA from 30.8% (control) to 36.5%, which were similar to the recoveries, 28.7% for control and 36.0% for high BCM dose, in the current study. Moreover, in their study, since total 2H recovery in SCFA and methane combined for nil BCM (control) and 10 μM BCM treatments were about 93% and 39%, respectively, it means 2HP converted into methane changed from 62.2% to 2.5% by addition of BCM. Similar to Goel *et al*. (2009), methane production was reduced by 91% and the recovery rate of 2HP into SCFA was significantly increased in the current study, which collectively demonstrates that the rumen microbiota adapted to reduced methanogenesis by redirecting the accumulated 2H into energy yielding SCFA. Moss *et al*. (2000) reported that methane production and the ratio (C2+C4)/C3 was highly positively correlated, and presumed that this relationship would be related to the flow rate of hydrogen into SCFA. A significant linear relationship between methane production and the (C2+C4)/C3 ratio was also observed in the current study.

H_2 production can be estimated from the ecological stoichiometry of rumen fermentation, with 90% of 2HP expected to be 2HU (hydrogen recovered as SCFA and methane) (Moss *et al* 2000, Faicheny *et al* 1999) under normal conditions. In this study, 2H recovered in methane and SCFA was calculated to be 40.8% at high BCM, which was similar to that (38-40%) of the *in vitro* study (Goel *et al* 2009*)*. However even though flow of 2H into SCFA increased by >20% at the high BCM dose, it was observed and calculated that the majority of 2H available from reduced methane formation flowed into H_2 gas instead of SCFA.

4.2 Monitoring of microbial populations

4.2.1 Methanogen and cellulolytic population monitoring

In the control period, the abundance of methanogens (1.36E+09 ±4.02E+08 copies of mcrA DNA/ml), *F. succinogenes* (5.98E+08 ±8.22E+07 copies of 16S rDNA/ml), *R. albus* (1.14E+08 ±1.90E+07 copies of 16S rDNA/ml), *R. flavefaciens* (8.23E+06 ±2.00E+06 copies of 16S rDNA/ml), and fungal biomass (29.38 ±7.94 µg/ml) was estimated using real-time PCR assays. The methanogen population showed a slight increase in abundance ($P<0.1$) for low BCM animals compared with the control and a significant decrease for the mid and high doses respectively ($P<0.05$, $P<0.01$) (Fig. 4). At the high BCM dose, the abundance of methanogens decreased by greater than 70 fold.
Populations of fibrolytic bacteria within the rumen were differentially affected by the decline in methanogens and subsequent increase in H_2 concentrations. *F. succinogenes* populations increased significantly ($P<0.05$) from the control period, however this may not be biologically significant as it did not equate to at least a two fold relative change (Fig. 4). *R. albus* was unaffected by the varying doses of BCM, while *R. flavefaciens* and the anaerobic fungal populations were decreased at all levels of BCM dosage ($P<0.001$).
There was no significant change in any of the four protozoal genera identified due to increases in BCM administration (data not shown).

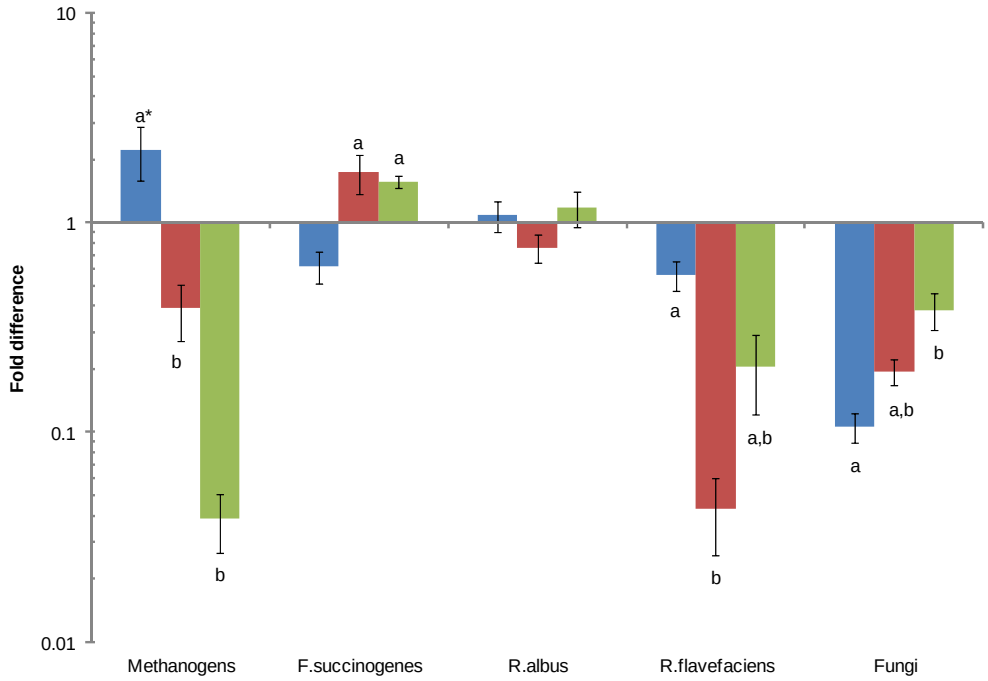


Figure 4. qPCR analysis *of m*ethanogens *F. succinogenes, R. albus*, *R. flavefaciens* and anaerobic fungi population changes in response to doses of BCM (Low,blue; Mid,red and High,green). *Letters denote significant differences from the control period, columns that do not share the same superscript for a species are significantly different to each other ($P<0.05$). Y axis denotes fold change from control period.

The population of the highly cellulolytic bacterium *F. succinogenes* increased slightly in relation to BCM treatment. Unlike the cellulolytic *Ruminococci* and fungal species in the rumen, *F. succinogenes* does not produce H_2 and is not susceptible to H_2 accumulation. Other fibrolytic bacteria and anaerobic rumen fungi rely on interspecies H_2 transfer for H_2 utilization and are therefore affected by the loss of the methanogens from this system. *R. falvefaciens* and *R. albus* are sensitive to partial pressures of hydrogen; increased hydrogen will inhibit NADH oxidation and divert hydrogen to the formation of succinate and ethanol away from acetate (Wolin *et al* 1997). This is less energetically favorable and cultures of *R. flavefaciens* grown without methanogens have a reduced capacity to degrade polysaccharides (Williams *et al* 1994). However, studies of cellulose degradation of *R. albus* with and without *Methanobrevibacter smithii* showed a small increase in ATP yield for the co-cultures but no difference in cellulose degradation (Pavlostathis *et al* 1990). These results along with recent fermentation studies of rumen microbial cultures with BCM (Goel *et al* 2009) are in close agreement with the microbial community changes in the current *in vivo* study except a decrease in the fungal population was not observed in the continuous culture system of Goel and coworkers (2009). In addition anaerobic fungal populations behave in a similar manner to the cellulolytic ruminococci in that co-culturing with methanogens results in a shift to actetate formation which is energetically more favourable and an increase in the rate and extent of cellulose degradation (Bauchop & Mountfort 1981). The protozoal population did not appear to be affected by BCM even though ecto- and endo-symbiotic methanogens associated with protozoa would have been inhibited. This result is also in agreement with the *in vitro* fermentation experiments of Goel and colleagues (2009).

4.2.2 16S rDNA/RNA "pyrotag" monitoring of microbial populations

Amplicon products of the expected size were observed for all goat microbiome samples, which were amplified using barcoded primers specific to each individual sample. After pooling and gel extraction of the PCR product, it was subjected to 454 sequencing on a ¼ of a plate and resulted in a total of 363,719 sequences. These sequences were quality filtered and separated into their original sample groups based on the detected barcode sequence with an average number of 8,042 reads per sample. After grouping sequences into operational taxanomic units (OTU) at 97% similarity the resulting data set was subjected to measures of alpha and beta diversity to determine the composition of the goat microbiome and its changes with respect to BCM treatment levels. Principal Coordinate Analysis (PCoA) of Beta diversity metrics based on entire sample microbiome data showed that the greatest variation in the data is explained based on the template amplified, either cDNA or gDNA (Figure 5). For both templates the next level of variation was explained via the treatment type (Figure 5). As the template is likely to represent different measures of the microbiome, in that gDNA is likely to indicate abundance of a population and cDNA indicate the activity of a population, further analysis was performed on each template as a separate data set.

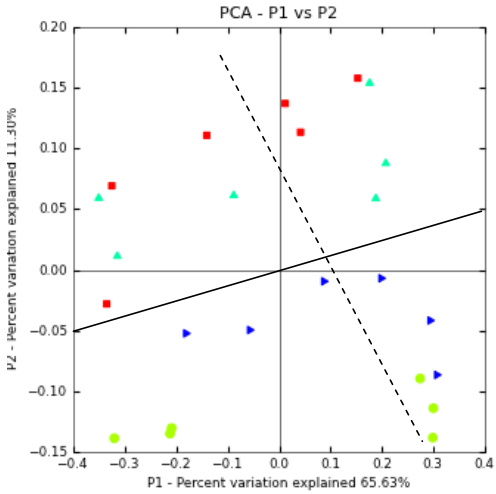


Figure 5. Principle Coordinate Analysis comparing changes in microbial OTU classification based on weighted Unifrac calculations for three animals at Control (red), Low (light green), Mid (dark blue) and High BCM (green) periods. Points to the left of the dashed line are generated from cDNA template, points to the right of the dashed line are from gDNA template. Solid line shows separation between Control/Low and Mid/High BCM.

Alpha diversity analysis showed that for both templates, the control and low BCM treatments allowed for the greatest diversity of bacteria within the rumen. A shift towards a more refined population was evident as the BCM concentration was increased with a reduction of 22% and 36% in estimated diversity for gDNA and cDNA, respectively (Figure 6). A list of the assigned taxonomies at the genus level, indicating their contribution to each treatment is supplied as an appendix (appendix 1).

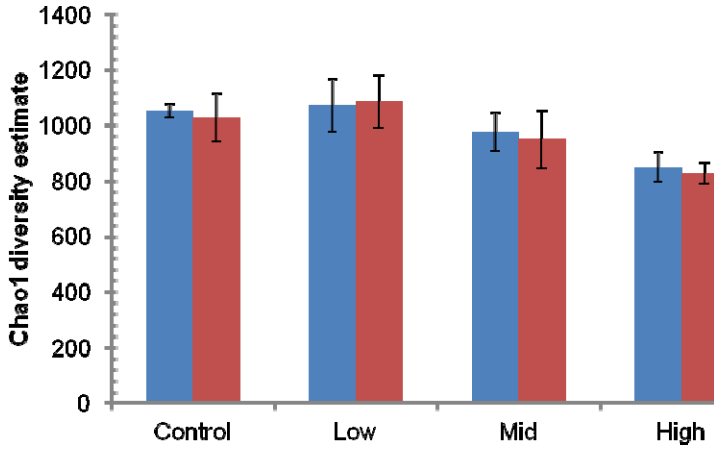


Figure 6. The mean Chao1 diversity estimates for microbial OTUs at control, Low, Mid and High doses of BCM. Blue bars represent estimates for gDNA template, red bars indicate cDNA estimates.

Calculation of distance measures between each OTU and subsequent PCoA of these data identifies the OTU's exhibiting the greatest variance between the templates and treatments (Figure 7.) OTU's assigned to Prevotellacae and Porphyromonadaceae families were found to contribute the largest numbers and or have shifted with respect to the high BCM gDNA samples. These observations were further supported by statistical analysis of the shifts in OTU abundance when performed using analysis of variance (ANOVA) calculations (Appendix 2). The same data shows that OTUs assigned to the families Porphyromonadaceae, Veillonellaceae (genus:*Selenomonas*) and Spirochaetaceae (genus:*Treponema*) are more abundant and show the greatest shifts for the high BCM cDNA samples.

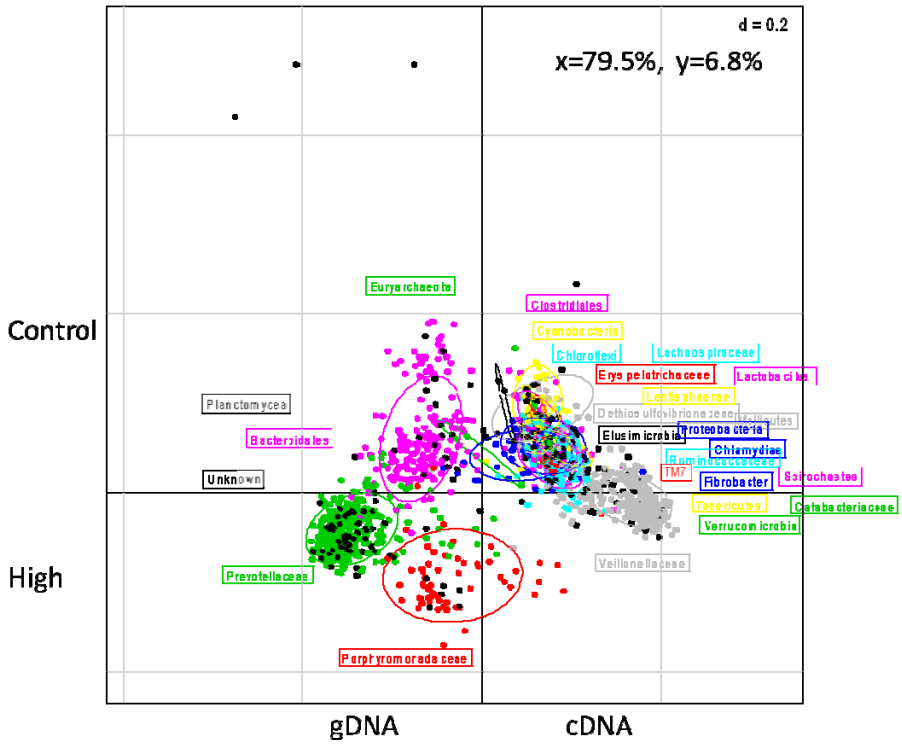
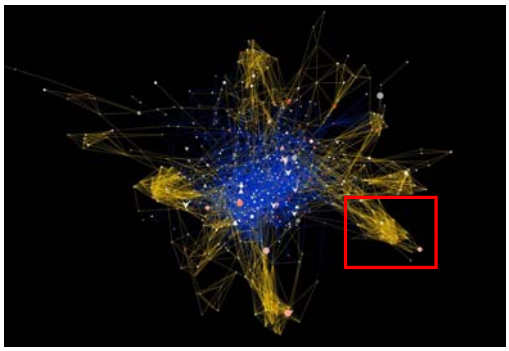


Figure 7. Principle Coordinate Analysis placing microbial OTUs with respect to template (gDNA, cDNA) and treatment (Control and High BCM).

Association networks based on the co-occurrence of the different OTUs among the data can be constructed; the resulting networks identifies relationships (negative and positive) among members of the microbiota. The association networks were generated using an association threshold of 1% based on Pearson correlation coefficients (Figure 8). When overlaid with taxanomic data and abundance fold change between treatments it is evident which groups of bacteria are responding in a similar manner to the increase in BCM dosing and the concurrent increase in available hydrogen within the rumen. Both OTU analysis and qPCR data conclude that *F. succinogenes* increases in abundance at higher concentrations of BCM. The highlighted sub-network shows the species of *Fibrobacter, Porphyronad, Prevotella, Selenomonas* and *Treponema* create a defined positive association network and are all found to be more abundant in High BCM. The identified groups within this network are capable of the breakdown of carbohydrates with concurrent conversion to propionate as a final end product, so in this case this sub-network has defined a propionate-producing sub-group of bacteria. Within this association network are several species that negatively associate with the propionate group consistently across the treatments. The most dominant negatively correlated speciecs within this sub-network is a *Victivallis sp.* The OTU was found to be distantly related to *Victivallis vadensis* a human faecal isolate that can grow on cellobiose or glucose producing acetate, ethanol, hydrogen and bicarbonate. Although when grown syntrophicaly with *M. hungatei* the *V. vandensis* converted

glucose exclusively to acetate and H_2. This bacteria does not produce propionate and based on its abundance in the high BCM treatment is not part to the propionate network but is rather consistently down regulated due to the higher hydrogen concentrations.

A



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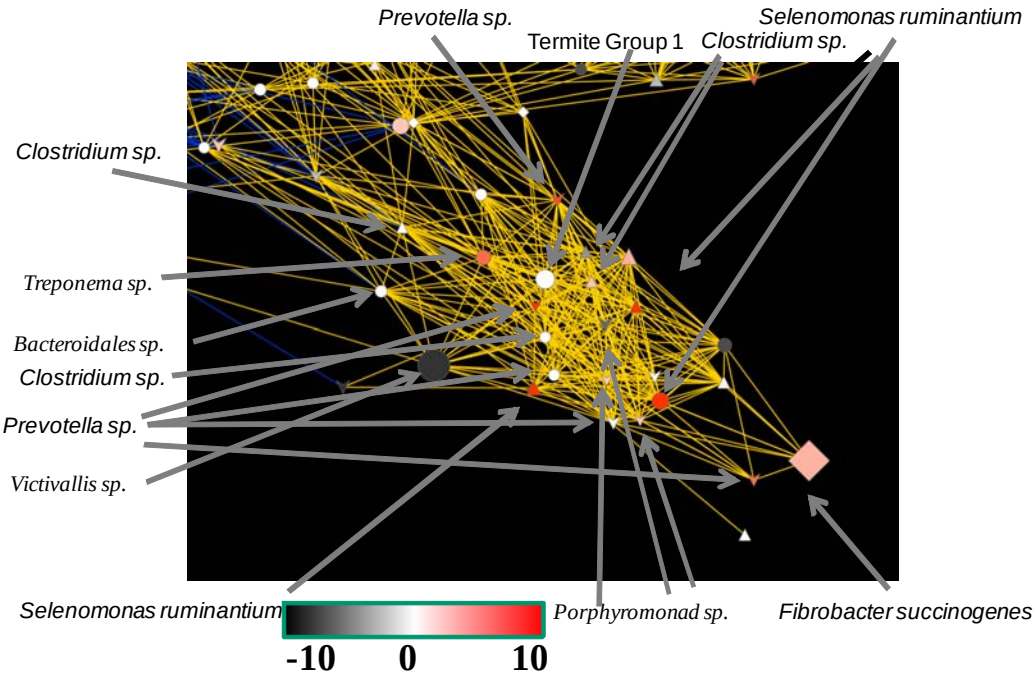


Figure 8. A) Association OTU networks for cDNA template, with OTUs represented as nodes and positive associations shown as gold lines and negative associations between nodes shown as blue lines. Highlighted boxed area is shown in B. Shape of OTU node designates Family level classification, size of node represents relative abundance of OTU to the goat microbiome, colour of OTU node indicates relative fold change from control (black) to high BCM (red) with scale indicated.

4.2.3 Development of real time PCR assay

Sequences representing statistically abundant OTU's for the gDNA high BCM were loaded into a 16S rDNA ARB database for higher resolution pyhlogenetic placement. Three OTU clusters were associated to known *Prevotella ruminicola* isolates (group 1, 7 and 12).
Prevotella group 1 and 7 specific qPCR primers were designed and validated against full length 16S rRNA sequences found in the ARB database. *Prevotella* group 1 comprised sequences from DGGE bands one to three and *Prevotella* group 7 comprised sequences generated from DGGE bands 7 and 8 (DGGE data shown in appendix 3). *Prevotella* group 1 was most similar (99%) to the near full length rumen clone F24-B12 (GenBank accession number, AB185591), while *Prevotella* group 7 primers were most similar (100%) to a near full length uncultured rumen bacterium clone L102RC-4-F10 (GenBank accession number, HQ399809). Results from using probe match analysis at RDP II and within ARB both showed the primers to be specific for their respective targets. PCR amplification of goat rumen microbial DNA using the specific *Prevotella* primer sets produced amplicons of expected size. Cloning and sequencing of these amplicons revealed all products examined, to align with their respective cluster with a similarity of greater than 98.5% to each other (data not shown). Primers for group 12 were also designed, but were found to be less specific as several clones were grouping outside of this cluster.
For both *Prevotella* groups there was a significant increase in numbers as measured by qPCR with respect to BCM dosing with group 1 increasing approximately 6 fold at the mid and high dose of BCM and cluster 7 increasing 2.5 fold at the highest BCM dosing compared to the control period (Figure 9). This exhibits a strong degree of correlation with the results observed for the increase in abundance of these OTUs in the "pyrotag" data and intensity for their respective DGGE bands. These results along with the sequence OTU abundance data show that *Prevotella* species are the predominate microbes shifting in these environments due to the dosing levels of BCM and subsequent increase in rumen hydrogen levels. Furthermore, the analysis of the rumen fermentation end products showed that there was a significant increase in propionate production. ***P. ruminicola*** produces propionate as an end product of fermentation using the randomizing (succinate) and non-randomising (acrylate) pathway from lactate (Wallnöfer and Baldwin 1966). Although, it was observed that the lactate concentrations for the treatments were not significantly different, two animals showed a decline in lactate as they transitioned from control to high BCM, suggesting the presence of this pathway within the system or the re-direction of lactate to pyruvate a common branching point between the randomizing and non-radomizing pathways.

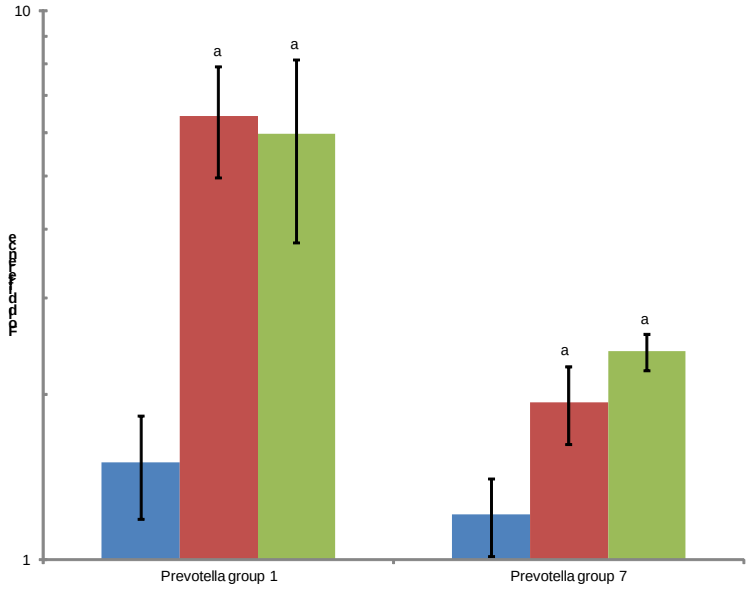


Figure 9. qPCR analysis of Prevotella cluster population changes in response to doses of BCM; Low (blue); Mid (red); High (green). [a] Significantly different from control period, ($p < 0.05$). Y axis denotes $\log_{10}$ fold change from control period.

Several other populations of interest exhibited shifts in their abundance due to administration of BCM, most notably were OTU's identified associating with *Succiniclasticum* and *Selenomonas* species. qPCR primers were designed to target one *Succiniclasticum* group and three *Selenomonas* groups. Primers were validated using clone libraries from rumen samples and in all cases the products were found to be specific for the primer set used (data not shown).

qPCR assay data showed significant increases in all the species at the highest BCM dosing compared to the control, while only two species were significantly increased at the mid dosing levels (Figure 10). *Succiniclasticum* and *Selenomonas* species are known to produce propionate as a major fermentation end product. *Succiniclasticum ruminantium* can grow on succinate as a sole carbon source to produce propionate using the randomizing pathway (van Gylswyk 1995), while *Selenomonas ruminantium* is known to be able to ferment lactate to propionate also via the randomizing pathway (Paynter and Elsden 1970).

Based on these data, the major shift in bacterial population abundance in response to BCM can be attributed to *Prevotella, Succiniclastium* and *Selenomonas* sp. utilizing pathways for propionate production which consume hydrogen via the randomizing (succinate) or non-randomizing (acrylate) pathways through the fermentation of sugars and lactate, respectively (Bryant *et al* 1958, Strobel 1992, Purushe *et al* 2010). It is likely that these pathways were the primary routes for consumption of hydrogen, which accumulated as a consequence of reduced methanogenesis. The development of qPCR primers to monitor two of these *Prevotella* clusters confirmed the observations of the DGGE analysis and 16S OTU data, in that these were dominant bacterial populations that increased in

abundance at high BCM dosing levels. However other bacteria such as *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Succinimonas amylolytica*, *Propionibacterium acnes* and *Veillonella parvula*, were observed to change and may also be involved, (Wolin *et al* 1997, Hungate 1966, Marounek *et al* 1989, Stewart *et al* 1997). Further studies involving deep tracer experiments with C-labelled sugars and lactate are required to determine the relative contribution of these pathways as the rumen microbiota adapts to high hydrogen concentration in the rumen.

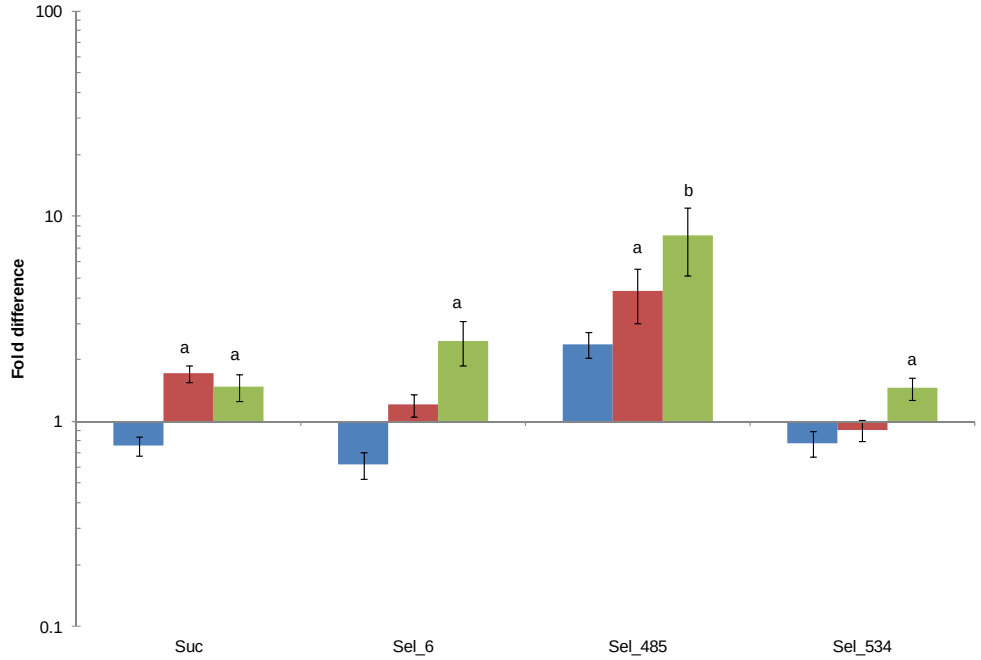


Figure 10. qPCR analysis of *Succiniclasticum* (Suc) and *Selenomonas* (Sel_6, Sel_485 and Sel_534) cluster population changes in response to doses of BCM; Low (blue); Mid (red); High (green). * Significantly different from control period, ($p < 0.05$). Y axis denotes $\log_{10}$ fold change from control period.

4.3 Functional gene analysis

Functional gene analysis

Gene libraries encoding functionial genes associated with methanogenesis (methyl coenzyme-M reductase, mcrA) and reductive acetogenesis (acetyl CoA synthase, ACS; formyl tetrahydrofolate synthetase, FTHFS) were constructed from control and high BCM samples.

Diversity of methanogens from the control goat rumen was similar to those observed in the cattle rumen in that they were dominated by members of the Methanobacteriales family. The administration of BCM produced a marked decrease in the methanogen diversity compared to the control period with the predominate OTU found to be affiliated with *Methanobrevibacter ruminantium* (Figure 11). In conjunction with the observed decrease in total methanogen numbers based on qPCR data, there is

also an observed decrease in methanogen diversity. Unlike in the cattle rumen there was no observed shift towards methanogens from the rumen cluster C grouping after dosing with BCM (Denman *et al.* 2007).

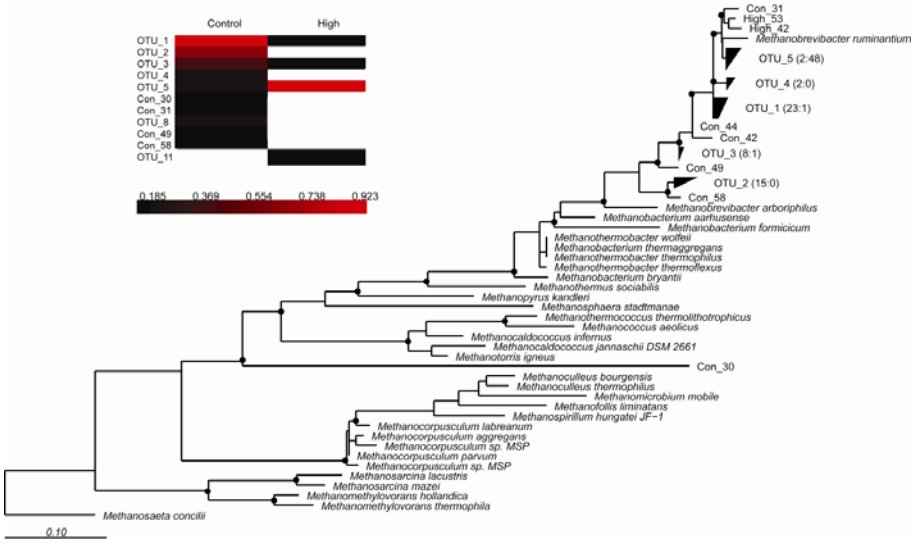


Figure 11. Phylogenetic placement of mcrA clones (maximum likelihood). Numbers in brackets indicate the contribution to the OTU from control or BCM respectively. Black circles at nodes represent a bootstrap value > 50%. Insert of heatmap showing abundance of sequences in a given OTU.

With an increase in ruminal H_2 concentrations reductive acetogens can compete successfully with methanogensis for energy within the rumen (Ungerfeld & Kohn 2006). In this regard an investigation into key functional genes from the reductive acetogensis pathway was undertaken. Firstly the well studied formyl tetrahydrofolate synthetase (FTHFS) gene was amplified from rumen samples. Richness (a measure of microbial diversity) of the FTHFS libraries between the samples was similar, however, the populations that make up the libraries was significantly altered (Figure 12). FTHFS is not restricted to reductive acetogens in the biosynthesis of acetate but also is involved in the biosynthesis of amino acids and pyrimidines. However, the use of a homoacetogen similarity scores (HSS) can be used to classify sequences as to their likeliness to originate from homoacetogenic bacteria (Henderson *et al.* 2010) Sequences with a HSS >80% were found to lie in the acteogenic clustering of the FTHFS phylogenetic tree (shaded region). From this region the predominant FTHFS OTU's for the control sample were positioned close to *Ruminococcus obeum,* while for BCM the most predominate OTU grouped closest to *Clostridium magnum* which was also observed in the control period at less abundance. Four OTU's comprising 43% of the sequences in the BCM sample were associated with the mixotrophic acetogen *Sporomusa* sp, while only a single representative from the control library was observed here (Figure 12). The ability of *Sporomusa* species to grow more productively on organic substrates such as methanol and lactate in conjunction with H_2 oxidation to

produce acetate gives these species an advantage over purely reductive acetogens (Breznak & Blum 1991). Although, this is mainly hypothesized as an ability to survive better under substrate limiting conditions, this may allow them to better adapt to changed environmental conditions.

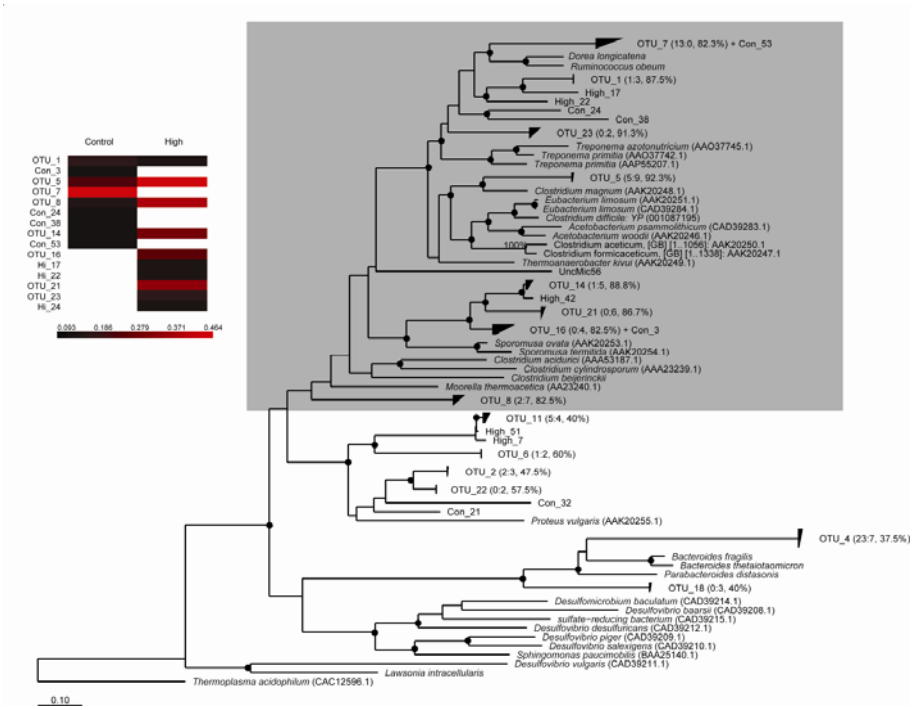


Figure 12. Phylogenetic placement of FTHFS clones (maximum likelihood). Numbers in brackets indicate the contribution to the OTU from control:BCM respectively. Black circles at nodes represent a bootstrap value > 50%. Shaded region indicates acetogenic bacteria clusters. Insert of heatmap showing abundance of sequences in a given OTU for sequences with a HSS > 80%.

Existing tools targeting the FTHFS gene are compromised by lack of specificity due to the involvement of FTHFS in other pathways. Acetyl-CoA synthase (ACS) is unique to the acetyl-CoA pathway and is an excellent marker gene for detecting acetogenic bacteria. However, as this pathway is also used by some methanogens and sulphur reducing species for generation of cell carbon and/or acetoclastic growth some caution in interpretation is still required (Ragsdale 1991). The presence of

ACS is a better indicator for the acetyl-CoA pathway than is FTHFS alone. Therefore,ACS libraries were generated using newly designed primers that exclude sulphate reducing bacteria and archaeal ACS genes. A greater diversity of sequences was collected from the control samples, and the diversity of the BCM sample tended to be a subset of the control sample. The most abundant OTU was the same for both samples being most closely associated to *Acetitomaculum ruminus*, although, the rank abundance of the OTU's was different between the samples (Figure 13). Within the BCM sample the majority of the sequences were associated with this *A. ruminus* grouping. This grouping was not identified using the FTHFS gene analysis due to the inability of the FTHFS primers used to amplify the gene from these species (Henderson *et al.* 2010). In addition the lack of congruency for the grouping of FTHFS genes associated with the *Sporomusa* species and ACS sequences can be explained in that this grouping could not be supported by bootstrapping for these species when comparing between FTHFS and ACS libraries and is therefore likely to be placed inaccurately within the ACS tree (Gagen *et al.* 2010).

Figure 13. Phylogenetic placement of ACS clones (maximum likelihood). Numbers in brackets indicate the contribution to the OTU from control:BCM respectively. Black circles at nodes represent a bootstrap value > 50%. Insert of heatmap showing abundance of sequences in a given OTU.

Functional gene analysis for methaogenic populations shows a restricting of the diversity with respect to BCM treatment and that the final population comprises a sub population of the initial methanogenic species. Reductive acetogens are hypothesised to be able to fill the vacated niche left by the methanogenic populations, and monitoring this function using the FTHFS or ACS marker genes for reductive acetogensis produced a similar effect to that of the methanogenic marker with a restriction of the diversity to a key sub-population. No accurate qPCR assay has been developed, so the quatitative assessment of these genes is still not available and it is therefore not apparent as to whether this function is increasing within the perturbed rumen.

4.4 Metagenomic data analysis of goat microbiome

Genomic DNA from all three animals was pooled together based on sampling period and one half of a plate each of 454 titanium sequences was performed for the control and high BCM sample. After removing replicated sequences generated during 454 library preparation each sample produced greater than 500 000 unique reads that were used in separate assembly runs (Table 5). Replicated sequences accounted for 19% and 22% of the control and high BCM metagenomic dataset respectively, which falls within the generally observed range of 15% to 35% (Gomez-Alvarez *etal* 2009). Over 6500 contigs greater than 500bp were generated for the control metagenome, while in excess of 9000 contigs were generated for the High BCM metagenome. The High BCM assembly generated a contig that was close to 40 kb in length, while the largest contig for the control was just over 23.5 kb in size (Table 5). The larger contig size and greater N50 values found in the High BCM assembly indicate the predominance of this species or a closely related relative. Annotation of the largest contig identified the sequences belonging to previously described genes from *Prevotella* species. The overall low N50 values and small contig sizes for both libraries is explained by the large diversity of bacterial species in the samples and indicates an incomplete coverage of the microbial genomes present. Any assembled contigs are likely to be representative of genes from predominant members or genes that are highly replicated in a given genome.
Hidden Markov models were used to identify and extract sequences containing 16S rDNA data, which could then be assigned to a phylogenetic grouping. Comparisons between the 16S OTU data generated from "pyrotag" sequencing and the metaegenomic data revealed a strong correlation indicating that the genomic content captured was representative of the pyhlogenetic groups identified previously (Appendix 4).

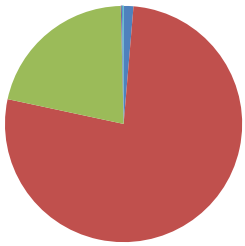
Table 5. Metageneomic assembly metrics

	Control	High_BCM
Orginal reads	633256	656433
De-replicated reads	512772 (81 %)	509928 (78 %)
16S genes	435	700
Contigs > 500 bp	6670	9393
All contigs	23508	18556
Largest Contig (bp)	6434	39361
Average contig length (bp)	671	1005
N50	636	1011
Average GC %	39	43

Classification of coding genes against an annotated non-redundant protein data base was performed at the MG-RAST server (http://metagenomics.anl.gov/). A broad summary of the findings show a difference between the two metagenomic communities. A reduction in reads assigned to archaeal genomes in the BCM sample was observed and coincides with the reduction of measured methane and methanogen numbers using gas analysis and qPCR respectively. There was an absence of phage associated sequence reads and a decrease in eukaryotic reads associated with fungal and protozoal

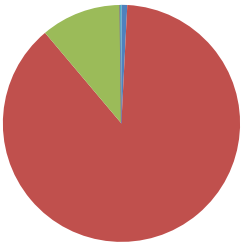
genomes in the BCM sample (figure 14, Appendix 4). This lead to a concurrent increase in reads being assigned to bacterial origin and reflects the constraining of the diversity due to the administration of BCM. Moreover a shift in the GC% for the sequences was observed in the BCM sample with an increase in reads with a GC% range of 40-70%. This would be in agreement with the observed increase in Bacteroidetes numbers, especially *Prevotella* groups which have a similar GC% content to their genomes.

Control



Archaea	4428 (1.3%)
Bacteria	255470 (77%)
Eukaryota	70509 (21.3%)
Viuses	532 (0.2%)
Other	748 (0.2%)

BCM



Archaea	3156 (0.8%)
Bacteria	349929 (88%)
Eukaryota	43530(10.9%)
Other	776 (0.2%)

Figure 14. Pie charts are based on the combined taxonomic domain information of all the annotation source databases used by MG-RAST.

Control

BCM

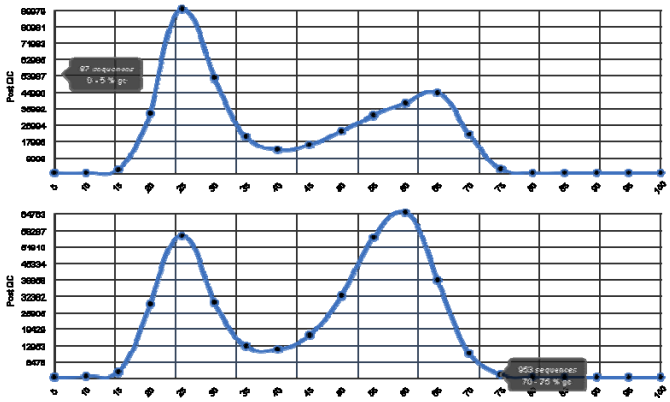


Figure 15. The histograms show the distribution of the GC percentage for the control and high BCM metagenomes. Each position represents the number of sequences within a GC percentage range.

A comparison of the changes at the phylum level for the metagenomic coding sequences was in strong agreement with the OTU generated data (Appendix 4). For most phyla not only was the change in abundance similar but the percentage of the data set represented by the phylum was also in agreement with the OTU data . A notable exception was the proteobacteria group which was assigned a ten fold greater proportion of reads in the metagenomic dataset relative to its apparent abundance estimated from the OTU analysis. There are two factors that are likely to contribute to this: 1) proteobacteria on average have larger genome sizes relative to other bacterial phyla and therefore on a molar ratio will contribute more to the metagenomic DNA pool that is sequenced; 2) Over 46% of microbial genomes currently sequenced are from proteobacteria and any annotation method that uses "best hit" matches are more likely to find a match with proteobacteria, especially for divergent bacterial species for which no closely related bacterial reference sequence is available. Although, it is important to consider that the assignment is based on the best comparison within the database and may only be accurate to an order or family level. Annotation to a rumen relevant set of microbial genomes would generate higher precession taxonomic assignment. Higher resolution phylogenetic assignment could be performed using computer algorithms that cluster sequences based on their nucleotide kmer patterns after training with relevant microbial DNA data. Regardless of this, the data still provides important information with respect to which broad groups of microbes possess the potential to perform specific tasks within the rumen. In particular, a closer investigation of the major pathways responsible for the production of propionate revealed that many groups of bacteria possessed genes involved in these pathways (Figure 16 and Appendix 5).

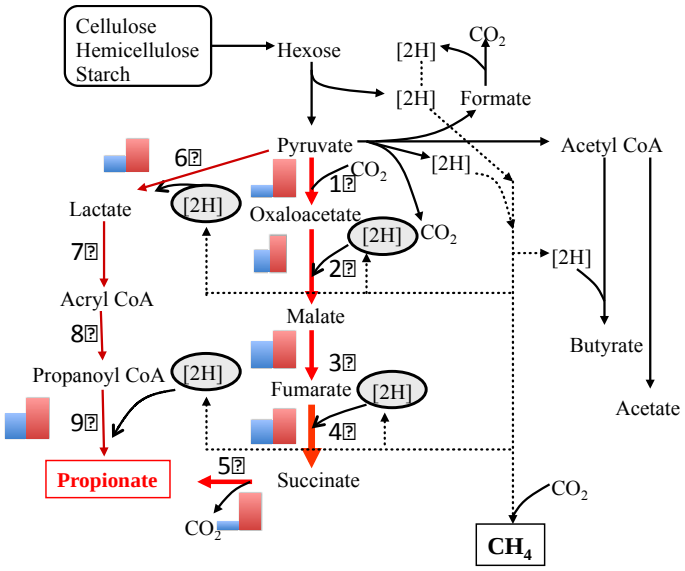


Figure 16. Microbial fermentation pathway for propionate production which consumes hydrogen via the randomizing (succinate, steps 1-5) or non-randomising (acrylate, steps 6-9) pathways through the fermentation of sugars and lactate respectively. Numbers refer to enzymatic conversion catalysed by 1; methylmalonyl-CoA-carboxyltransferase (EC 2.1.3.1), 2; Malate dehydrogenase (EC 1.1.1.37), 3; fumarase (EC 4.2.1.2), 4, fumarate reductase (EC 1.3.5.4), 5; propionyl-CoA:succinate CoA transferase (EC 2.8.3-), 6; D-lactate dehydrogenase (EC 1.1.1.28), 7; lactyl-CoA dehydratase (EC 4.2.1.54), 8; acryloyl-CoA reductase (EC 1.3.99.3), 9: propionate CoA-transferase (EC 2.8.3.1). Bar charts indicates percentage of metagenomic reads assigned with the described function for control (blue) and High BCM (red).

Only two genera, *Prevotella* and *Selenomonas* bacteria could be confidentially assigned for all enzymes involved in the randomizing succinate pathway. Both of these bacterial groups were also assigned lactate dehydrogenase activity, which converts pyruvate to lactate for use in the non-randomizing acrylate pathway (Appendix 5). Neither lactyl-CoA dehydratase or acryloyl-CoA reductase were found in either of the metagenomic datasets, indicating that the non-randomizing acrylate pathway is not the major route for the production of propionate in the rumen or at least not contained within the abundant bacterial groups. As lactate dehydrogenase is a reversible reaction it is likely that these same bacteria are capable of converting lactate back to pyruvate for use in the randomizing succinate pathway. The percentage of metagenomic sequences assigned to each enzymatic step for the randomizing succinate pathway for the control sample were of similar abundance, however, the conversion of succinate to propionate was considerably less observed suggesting that the abundance of bacteria capable of taking succinate through to propionate are less then those that produce succinate as an end product (Figure 17). For all enzymatic steps in propionate production a greater percentage of the metagenomic reads was assigned to the BCM treatment further supporting the increase in this pathway for the sequestration of hydrogen.
The presence of these pathways is a good indication that these populations are responsible for the shift in microbial fermentation products.

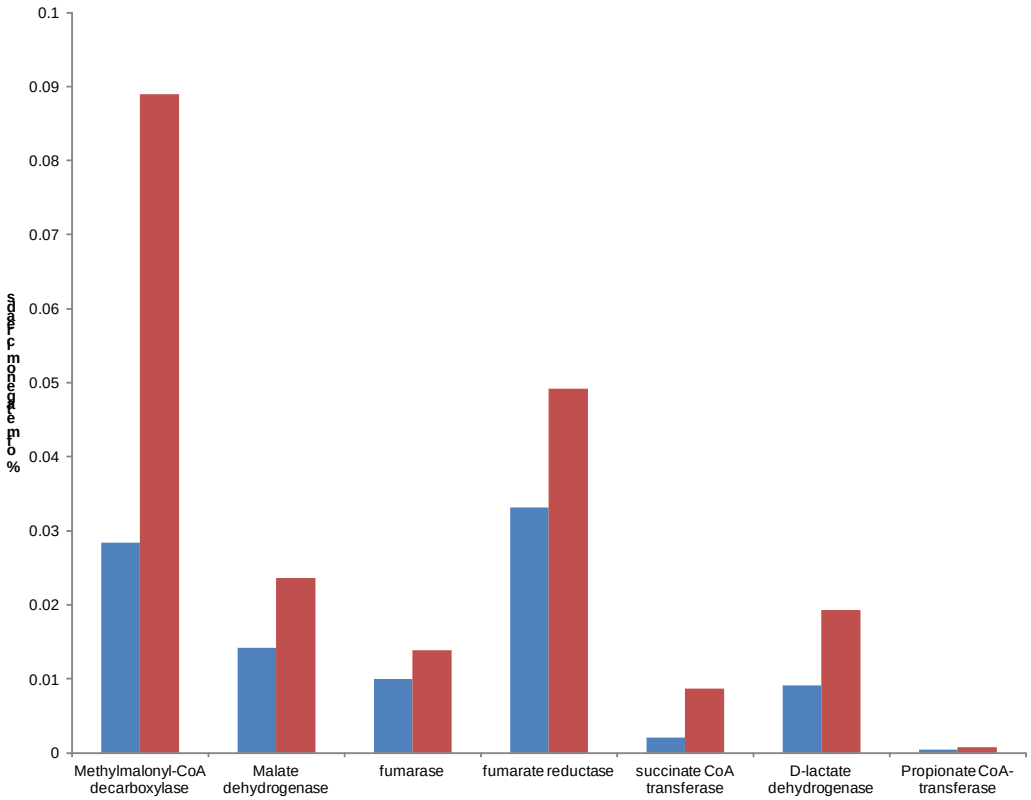


Figure 17. Percentage of metagenomic sequencing reads that assigned with enzymatic function for propionate production.

Carbon monoxide dehyrogenase/Acetyl CoA synthase (CODH/ACS), corrinoid/Fe-S:methyl-transferase and the corrinoid/Fe-S protein itself are enzymes that are unique to the reductive acetogenic pathway and possibly provide the best evidence for reductive acetogenic bacteria. Only limited sequence data was obtained for these key enzymes from both metagenomic libraries emphasizing relatively low abundance of bacteria capable of reductive acetogensis (Table 6). All enzymes were most confidently assigned to the Clostridiales class of bacteria. The data although limited also shows a reduction in the identification of these key enzymes in the BCM treated rumen. Bromochloromethane is a halogenated methane analogue that is believed to inhibit methane production by reacting with reduced vitamin B12 and inhibiting the cobamide-dependent methyl transferase step of methanogenesis (Wood et al., 1968; Chalupa, 1977). Similar structured corrinoid methyltransferases are key to reductive acetogenesis and there is no evidence to date as to the effect BCM exhibits on reductive acetogenic bacteria. There is a need for these experiments to be performed on rumen reductive acetogenic isolates to understand the effects that such an inhibitory compound may elicit.

Table 6. Indicative reductive acetogenic enzymes

Enzyme	*Control* (%)*	*BCM (%)*
CO dehydrogenase/acetyl-CoA synthase complex	0.000947484	0.000609
methyltetrahydrofolate:corrinoid/iron-sulfur protein	0.000473742	0.000152
corrinoid/iron-sulfur protein	0	0.000152

* Values are percentage of metagenomic library sequencing data that was assigned the function.

4.5 Metatranscriptomic data analysis of goat microbiome

Microbial total RNA from all three animals was pooled together based on sampling period and one lane each of illumine HiSeq was performed for the control and high BCM samples. After quality filtering the control lane contained 148 million paired reads and a 10% failure of reads, while the BCM lane contained 88 million paired reads with a 7% failure rate (Table 7). Ribosomal sequences were identified from within the total RNA sequencing data and removed leaving approximately eleven million paired reads per library which was 7% and 13% of the original sequencing data respectively for the control and BCM libraries.

Table 7. Meta-transcriptomic sequencing metrics

	Control	%	BCM	%
Raw reads	164,195,270		94,892,460	
Quality passed (pairs)	148,694,856	90.6	88,125,502	92.9
Quality passed (singles)	7,044,296		3,139,010	
rRNA containg reads	137,830,036		76,856,974	
Non-ribosomal reads	10,864,820	7.3	11,268,528	12.8
Mapped to MG				
No Hits to MG data	9,633,175		9,062,752	
CDS hits to MG data (counts)	1,231,645	11.3	2,205,776	19.6
CDS hits of total MG	125,374	16.9	15,386	2.1

The previously generated metagenomic data sets were concatenated together to generate a single database of gene scaffolds for the mapping of the mRNA sequence data. Eleven percent of the control RNA library's non-ribosomal sequences mapped to the metagenomic database accounting for 125,000 separate targets from the metagenomic dataset. The assigned phylogeny of this data when considered with the OTU RNA analysis concludes that there is a large diversity of microbes that are metabolically active within the control sample even when accounting for the low coverage and variation between the metagenomic and metatranscriptomic data sets (Appendix 6). Approximately 20% of the BCM non-ribosomal sequences mapped to a total of just over 15,000 metagenomic

sequences. For the BCM meta-transcriptome the restriction in OTU diversity is also reflected in the transcriptome as nearly 20% of the sequence reads could be identified to a restricted set of genes compared to the control set.
The number of RNA sequences that hit a given coding sequence were recorded for each RNA library and the numbers were then normalised for the variation in the library size before differential expressed genes were identified (Figure 18). A smear plot based on the calculated log fold change against the log concentration for the target gene produces a "smear" of data points at the left most edge that accounts for data that has a zero for one of the treatments. This is done to compensate for the fact that for a zero value the log- fold change is technically infinite, and the log-concentration is negative infinity. Although the plot is slightly artificial it allows for all data points to be visualized in the one plot. The "up-regulated" genes for the BCM treatment were associated with cell division and central metabolism pathways and mostly associated with *Prevotella, Paluibacter* and *Selenomonas* metagenomic assigned reads. We know that these populations are more abundant based on our 16S rDNA analysis, however as some of these also predominate the metagenomic samples the chance of these mapping are increased. Regardless, of this proposed bias this identifies the metabolically active bacteria groups for this twenty percent of the data.

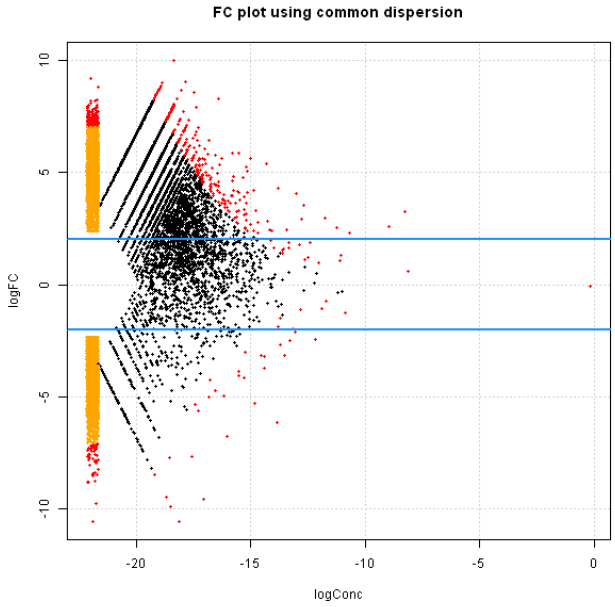


Figure 18. Differentially expressed transcripts with respect to control and BCM treatment. Positive logFC are with respect to up regulation for BCM treatment.

Transcripts associated with succinate and propionate production as part of the microbial central metabolism were identified and were also associated with the "up-regulated" species identified above

with respect to cell division and growth. Transcript mapping would indicate that at the time of sampling the major difference with the BCM rumen was a reduction in lactate being converted to pyruvate for use in the randomizing succinate pathway (Figure 19). Lactate dehydrogenase actitvity was nearly exclusively assigned to *Selenomonas* species, which are known to perform this function in the rumen (Paynter & Elsden 1970). For all transcripts both *Prevotella* and *Selenomonas* species are actively performing succinate and propionate fermentation. However, some transcripts were not accurately classified to the genus or family level, but where classified with the Bacteroidales order and were most similar to *Prevotella* sequences. As no metagenomic reads were assigned to the acrylate pathway, there was no genes to map these transcripts too if they were active in this system, therefore a *de novo* based approach would be required to elucidate the presence of this pathway.

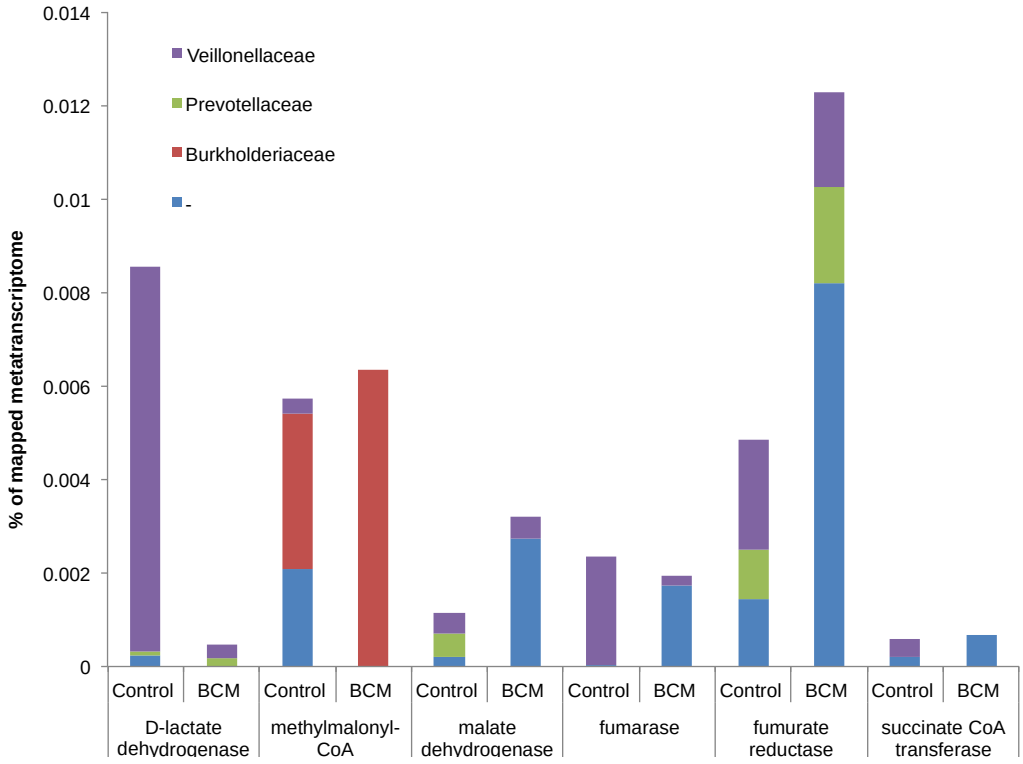


Figure 19. Percentage of metatransciptomic sequencing reads that assigned with enzymatic function for propionate production, classified phylogenetically to the family level.

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A second analysis of the coding data set was undertaken using *de novo* assembly techniques, which attempt to assemble reads into larger contigs without mapping to a reference database. Compared to the metagenomic mapping technique both libraries were able to use a greater percentage of their

reads increasing to 67%-76% for the control and 39%-66% for the BCM library depending on the kmer setting. This produced between 220 to 240 thousand contigs for the control sample and approximately 40 thousand contigs for the BCM library (Table 8). Again we observed a restriction in the number of transcripts for the BCM library confirming the up-regulation of specific bacteria and function.

Table 8. Meta-transcriptomic *de novo* assembly metrics

	Control	%	BCM	%
kmer 21				
Final_graph_nodes	2,709,108		128,199	
N50	100		86	
max-length	3,234		549	
Reads_used	7,909,959	72.8	7,492,808	66.5
mRNA	222,481		41,542	
Reads	8,257,710	76.0	7,401,971	65.7
kmer 31				
Final_graph_nodes	1,162,296		61339	
n50	117		97	
max-length	5,436		607	
Reads_used	7,332,133	67.5	5600425	49.7
mRNA	240,796		40,023	
Reads	7,249,841	66.7	4,375,568	38.8

Currently 10% of the *de novo* data have been annotated, further classification of the remaining coding sequences continues. Again the major propionate pathway functions are being assigned to *Prevotella* and *Selenomonas* sp., this is due to the method of taxanomic classification being assigned based on best hit to sequences to available in the public databases (Figure 20). Many Bacteroidetes sequences were not confidently classified past the phylum and order level and indications of sequences similarity suggest that they form a grouping closest to the described isolate *Paludibacter propionicigenes*. This bacterium is a known propionate producer which was isolated from rice stubble from an irrigated rice paddy in Japan (Ueki *et al* 2006). Further annotation to a rumen relevant set of microbial genomes would generate higher precession taxonomic assignment. In addition, higher resolution phylogenetic assignment will be performed using computer algorithms that cluster sequences based on their nucleotide kmer patterns.

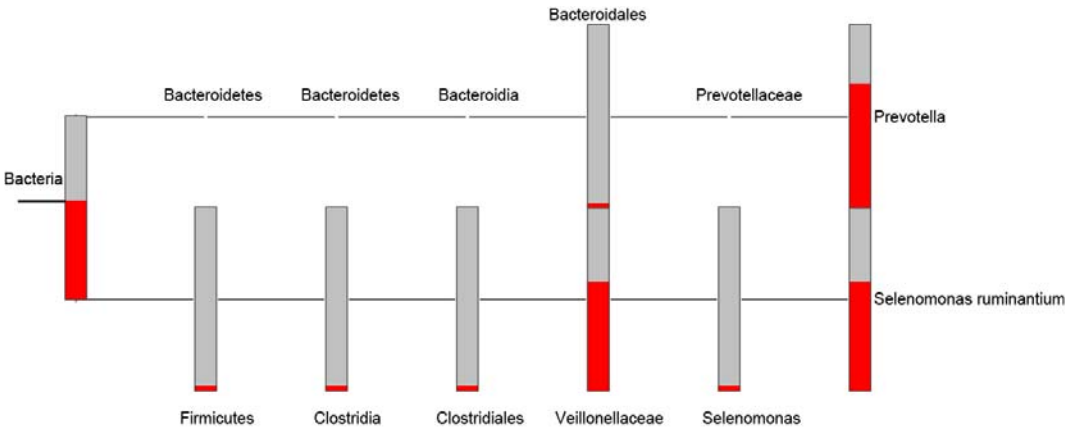

Figure 20. Metatransciptomic sequencing reads that assigned with enzymatic function for propionate production, classified phylogenetically. Red bar indicates the percentage of confidently assigned reads at each taxonomic level.

4.6 Bacterial isolations

Bacterial isolations were performed on rumen samples resulting in the isolation of six axenic cultures. These were presumptively identified based on their full length 16S sequences as two *Prevotella* spp. a Porphyromonadaceae and three Clostridiales (Table 8). The two *Prevotella* isolates were representatives from OTUs that were only slightly elevated in the BCM treatments compared to the control with respect to the gDNA template and cDNA, suggesting that their abundance and metabolic activity within the rumen is at a steady state regardless of the treatments. Two Clostridales isolates RM29 and RM58 were representatives of OTUs that were noticeable increased in the BCM treatments with a 4.6 and 11 fold change in abundance respectively. Although RM58 had the greatest fold change in abundance, at the RNA level this reduced to only a 4 fold change and as a whole this OTU was a minor contributor to the total RNA pool.

Table 9. Bacterial isolate and their representative OTU data

Isolate	OTU fold change		Taxonomy					
	gDNA	cDNA	Order		Family		Genus	
RM4	1.2	1.3	Bacteroidales	100%	Prevotellaceae	100%	Prevotella	100%
RM17	1.3	1.8	Bacteroidales	100%	Prevotellaceae	100%	Prevotella	100%
RM8	0.8	1.1	Bacteroidales	96%	Porphyromonadaceae	68%	Tannerella	32%
RM29	4.6	4	Clostridiales	100%	Incertae Sedis XIV	54%	Blautia	54%
RM58	11	3	Clostridiales	100%	Lachnospiraceae	100%	Roseburia	30%
RM66	0.8	0	Clostridiales	100%	Lachnospiraceae	100%	Coprococcus	70%

Detailed culturing studies to define the optimum growth conditions and end product formations should be undertaken to better understand the ability of these isolates to exploit the higher concentrations of hydrogen that will be available within the modified rumen.

5 Success in Achieving Objectives

1. *Establish a small ruminant model with and without the methane inhibitor (bromochloromethane) to promote metabolically active schemes active in the rumen that shift from methanotrophy and alternative pathways of hydgrogen consumption.*

   An animal trial was undertaken using four rumen fistulated goats at the National Institute for Livestock and Grassland Science in Japan. Methane and hydrogen production were measured in these animals using open-circuit respiration chambers while feeding the methane inhibitor bromochloromethane (BCM). The Japanese Institute (NILGS) has a unique capability of conducting in vivo measurements of methane and hydrogen in open-circuit respiration chambers and energy metabolism in large and small ruminants.

2. *Quantify the effects of reduced methanogenesis on rumen fermentation and digestion.*

   Measurement of methane production and rumen hydrogen concentration in animals were performed using open-circuit respiration chambers and hydrogen sensors within the chambers while feeding three levels of the methane inhibitor BCM. Measurements of digestibility were taken throughout the trial for the control period and the highest dose of BCM.

3. *Through the use of real time PCR assays and microarray analysis, quantify the effects of reduced methanogenesis on microorganisms that are known to be involved in fibre digestion and alternative hydrogen utilisation.*

   Rumen microbial DNA from the run animal trial was used for investigating microbial phylogeny and functional changes. Previously designed assays for the detection and monitoring of methanogenic and key fibrolytic populations were undertaken. Further analysis was performed through the design of new assays to monitor key populations that were identified through the use of "next generation sequencing of the goat microbial rumen samples. Taxonomic analysis of these microbial populations and identification of microbial populations that responded to the increase in H_2 concentration were performed using these new technologies over the originally proposed microarray methods due to the increase in resolution afforded to next generation sequencing methods.

4. *Through the use of metagenomic techniques determine the microorganisms and genes involved in hydrogenotrophy in response to reduced methanogenesis and higher concentrations of hydrogen.*

   Using "next generation sequencing rumen microbial DNA from the previously run animal trial was used for metagenomic sequencing and analysis of functional genes from the control and high BCM samples. Tools to identify the metagenomic sequences

and group them based on function were undertaken and further analysis of this data has allowed for the functional genes involved in hydrogenotrophy to be linked to their phylogenetic groups.

5. *Isolate microbes that are responsible for the alternative routes of hydrogenotrophy in the rumen.*

   Culture based experiments to isolate specific bacteria that were observed to change with respect to the increase in hydrogen within the rumen were undertaken. Isolated bacteria were then identified and several were found to be associated with important OTUs for the goat rumen. These bacteria are ready to undergo an more extensive characterization to better understand their capability to use hydrogen in competition with rumen methanogens

6. *Based on the outputs of the previous objectives, conduct an animal supplementation trial that demonstrates the optimal level of methane reduction in the rumen and response in alternative hydrogenotrophy to prevent detrimental effects on rumen fermentation.*

   This objective was achieved by modifying the first feeding trial when it was observed that the level of BCM was having an effect on methanogenesis with no detrimental effect on feed digestibly. A modification of the initially planned trial was made that allowed for the subsequent increasing of the administered BCM dose. Measurements were taken at all varying dose levels that indicated that rumen function with respect to fed digestion was not effect, while methane production was continually suppressed.

6 Impact on Meat and Livestock Industry – now & in five years time

Methane production from ruminants not only poses an environmental challenge to the producer but also represents a production loss to the animal. Identifying microbial populations that can re-direct hydrogen from methanogenisis or utilize the surplus hydrogen production in rumen systems where methanogenic species have been inhibited will allow for the development of intervention strategies that not only reduce methane but increase animal production. Key to this is a greater knowledge of the microbial populations that can perform this function and the "flow on" effects that their increase in abundance within the rumen elicits upon other microbial populations, especially those involved in fibre breakdown and energy production for the animal. The data generated from this project firstly shows that for animals on a restricted relatively good quality diet that methane emissions can be reduced to 90% with no detrimental effect to the animal even though rumen hydrogen levels were detected to rise dramatically. These data illustrate the robustness of the rumen microbial ecosystem to adapt to changes within the system and over the recording period showed partial redirection of the surplus hydrogen through microbial fermentation to SCFA products. A greater understanding of the responsible bacterial populations and the pathways they use in the future should allow for the defining of management strategies that target the promotion of these populations with feed supplements to not only reduce methane but stimulate re-direction of hydrogen into SCFA for animal growth.

The rumen is a rich and dynamic microbial ecosystem with primary species responsible for plant breakdown and their products used to sustain other microbial groups or directly taken up by the animal. Many microbial groups are reliant on other members of the rumen ecosystem to provide them with their nutrients and an environment suitable for them to survive and thrive in. This project has provided a greater understanding of the interactions between microbial species and will help us in the future to predict the effect that altering key populations will have on rumen function. By overlaying the genetic and functional potential of these groups we can not only identify these populations but define their roles within the ecosystem. These data sets would form an integral part of producing accurate rumen microbial models for better designing intervention strategies.

7 Conclusions and Recommendations

The findings of this research show a dose-dependent inhibitory effect of BCM on the goat rumen for methane production. Doses of net BCM, 0.04, 0.16, and 0.4 g/100 kg LW, reduced methane production by 5, 71, 91%, respectively, compared to controls and there was no effect on maintenance feed intake and NDF digestibility. This indicates fibre digestion was not compromised on a highly digestible diet even though H_2 concentration increased markedly in the rumen due to inhibition of methanogenesis. The metabolic association of methanogens with other gut microbes has an important role in fermentation of plant material in the herbivore gut. Under anaerobic conditions, hydrogen is formed by the oxidation of NADH to NAD+ (Wolin *et al* 1997) and consequently, when hydrogen accumulates, it can negatively influence the amount of NADH oxidation. Consequently, bacteria must then redirect fermentation towards schemes that produce more reduced forms of fermentation end products (e.g. lactate, ethanol), which also reduces the ATP yield and overall efficiency of bacterial growth and hydrolysis of substrates such as cellulose (Wolin *et al* 1997). Clearly here we find that although some fibrolytic species such as *R. flavefaciens* and anaerobic fungi were inhibited other species were promoted and net overall fiber digestion was not affected.

The suppression of methane production by BCM led to a large accumulation of H_2 while rumen fermentation end products were mostly unchanged except for significant increases in propionate and iso-valerate at the mid and high doses. However even though the flow of 2H into SCFA increased by >20% at the high BCM dose, it was observed and calculated that the majority of 2H available from reduced methane formation flowed into H_2 gas instead of SCFA. Therefore there still remains an opportunity to further enhance the 2HP flows into SCFA in order to reduce production of greenhouse gases and to increase feed efficiency in ruminants.

There was no change to acetate levels, which possibly indicates that reductive acetogenic bacteria have not contributed significantly to the consumption of the accumulated H_2. The acetate concentration, however, could reflect a decreased production of acetate by oxidative pathways combined with increased production from reductive acetogenesis. This could be resolved in future studies with the use of labelled substrate required for autotrophic growth of reductive acetogens.

Microbial ecology studies of the BCM affected rumen showed that the major shift in bacterial population abundances can be attributed to *Prevotella, Succiniclastium* and *Selenomonas* sp. These microbiota were found in assocaiation networks to form a sub-network that possessed the key function of carbohydrate breakdown with the con-current release of propionate as an end product. These species are capable of utilizing pathways for propionate production which consume hydrogen via the randomizing (succinate) or non-randomizing (acrylate) pathways through the fermentation of sugars and lactate, respectively[j]. It is likely that these pathways were the primary routes for consumption of hydrogen, which accumulated as a consequence of reduced methanogenesis.

The development of qPCR primers to monitor these populations confirmed the observations of the 16S OTU data. Metagenomic sequencing of the BCM rumen revealed an absence of phage associated sequence reads and a decrease in eukaryotic reads associated with fungal and protozoal genomes again indicating the sensitivity of these population to higher hydrogen concentrations.

In conclusion, this in-vivo study in goats showed that inhibition of methanogenesis by >80% dramatically increased ruminal H_2 concentration without affecting dry matter intake and feed

digestibility. A reduction in fibrolytic rumnococci and rumen fungi may have been compensated by a slight increase in *Fibrobacter* which prevented an adverse effect on fibre digestion. The methane-inhibited rumen appeared to adapt to the high H_2 levels by shifting fermentation to propionate which was mediated by an increase in the population of hydrogen-consuming *Prevotella* spp. As the rumen adapted to the high H_2 concentration the flow of metabolic hydrogen into SCFA increased by >20% but the majority of 2H (>80%) which is normally consumed in methane formation was expelled by the animal. Therefore consumption of this excess hydrogen into yielding substrates for the animal will require the provision of dietary supplements to drive hydrogen uptake or augmentation of minor hydrogenotrophic pathways such as autotrophic reductive acetogenesis. It was predicted that the accumulation of hydrogen from inhibition of methanogenesis would have a 'tipping point' at which rumen fermentation was compromised but this did not occur on a high quality diet. Although the rumen microbial ecosystem compensated for the increases in hydrogen without any detrimental effects on fibre digestion and animal production on a high quality diet responses on roughage diets typical of tropical production systems need to be tested.

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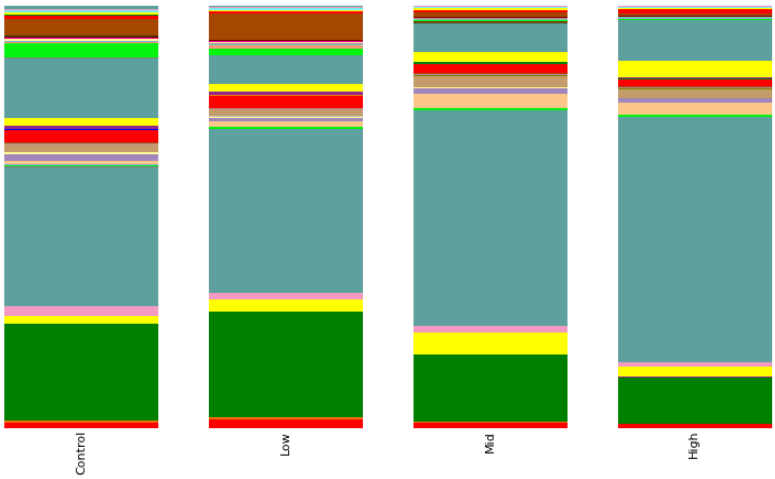
9 Appendices

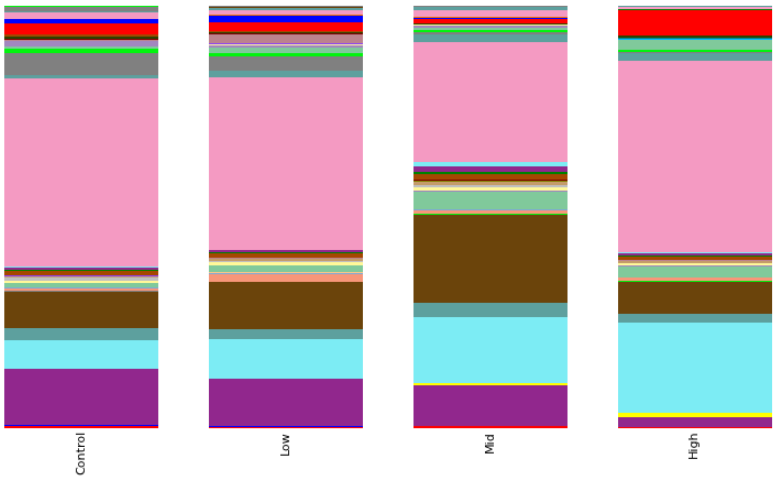
Relative abundance of OTU taxanomic assignment for treatments based on gDNA template.



		Total		Control	Low	Mid	High
Legend	Taxonomy	count	%	%	%	%	%
	No blast hit;Other;Other;Other;Other;Other	0	1.4%	1.3%	2.0%	1.2%	1.2%
	k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanobrevibacter	0	0.0%	0.1%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__WCHD3-02;g__	0	0.4%	0.5%	0.5%	0.5%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__	1	18.6%	22.8%	25.0%	15.8%	10.9%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__	0	0.1%	0.0%	0.0%	0.1%	0.2%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Paludibacter	0	3.0%	1.8%	2.9%	5.1%	2.3%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__	0	1.7%	2.3%	1.6%	1.6%	1.2%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	2	45.1%	32.8%	38.7%	51.0%	57.9%
	k__Bacteria;p__Chlamydiae;c__Chlamydiae;o__;f__;g__	0	0.0%	0.2%	0.0%	0.0%	0.0%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__SHD-231	0	0.1%	0.1%	0.1%	0.1%	0.1%
	k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__	0	0.4%	0.3%	0.4%	0.5%	0.4%
	k__Bacteria;p__Elusimicrobia;c__Elusimicrobia;o__Elusimicrobiales;f__Elusimicrobiaceae;g__	0	0.1%	0.2%	0.1%	0.0%	0.0%
	k__Bacteria;p__Elusimicrobia;c__Endomicrobia;o__;f__;g__	0	0.1%	0.1%	0.1%	0.0%	0.0%
	k__Bacteria;p__Fibrobacteres;c__Fibrobacteres;o__Fibrobacterales;f__Fibrobacteraceae;g__Fibrobacter	0	2.0%	0.7%	1.2%	3.3%	3.0%
	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0	0.0%	0.0%	0.1%	0.0%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	0	1.2%	1.6%	0.9%	1.3%	0.9%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Catabacteriaceae;g__	0	0.2%	0.4%	0.2%	0.1%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium	0	0.1%	0.1%	0.1%	0.1%	0.1%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__ClostridialesFamilyXIII.IncertaeSedis;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__ClostridialesFamilyXIII.IncertaeSedis;g__Eubacterium	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	0	2.0%	1.8%	1.6%	2.6%	2.1%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Butyrivibrio	0	0.1%	0.1%	0.1%	0.2%	0.1%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium	0	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	0	0.3%	0.3%	0.2%	0.3%	0.3%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Eubacterium	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Pseudobutyrivibrio	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Shuttleworthia	0	0.1%	0.0%	0.0%	0.1%	0.1%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	0	2.3%	2.9%	2.7%	2.1%	1.5%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium	0	0.2%	0.2%	0.2%	0.1%	0.2%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Eubacterium	0	0.0%	0.0%	0.1%	0.0%	0.0%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	0	0.2%	0.1%	0.1%	0.4%	0.3%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	0	0.5%	0.8%	0.7%	0.2%	0.2%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__	0	2.4%	1.8%	1.8%	2.3%	3.8%
	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Acidaminococcus	0	0.0%	0.0%	0.0%	0.0%	0.0%

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Propionispora	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Selenomonas	0	9.3%	14.1%	6.7%	6.8%	9.7%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Thermosinus	0	0.2%	0.1%	0.2%	0.3%	0.2%
k__Bacteria;p__Lentisphaerae;c__Lentisphaerae;o__Victivallales;f__;g__	0	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Lentisphaerae;c__Lentisphaerae;o__Victivallales;f__Victivallaceae;g__	0	1.3%	3.4%	1.5%	0.3%	0.1%
k__Bacteria;p__Lentisphaerae;c__Lentisphaerae;o__Z20;f__;g__	0	0.3%	0.4%	0.6%	0.2%	0.1%
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__;f__;g__	0	0.2%	0.2%	0.4%	0.1%	0.2%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Rhodopseudomonas	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__;g__	0	0.2%	0.5%	0.3%	0.1%	0.0%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfuromonadales;f__Geobacteraceae;g__Geobacter	0	0.1%	0.1%	0.2%	0.1%	0.0%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Polyangiaceae;g__	0	0.0%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__SR1;c__;o__;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__MiNP2-04;o__;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Sphaerochaetales;f__Sphaerochaetaceae;g__Sphaerochaeta	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Spirochaeta	0	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema	0	0.3%	0.2%	0.2%	0.3%	0.3%
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__Pyramidobacter	0	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__TG5	0	3.0%	4.2%	6.2%	1.2%	0.5%
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__F16;g__	0	0.6%	0.8%	0.4%	0.4%	0.9%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__	0	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Bulleidia	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__p-75-a5	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__vadinHA31;g__REN20	0	0.4%	0.5%	0.4%	0.5%	0.2%
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae;g__Anaeroplasma	0	0.4%	0.5%	0.4%	0.3%	0.4%
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39;f__;g__	0	0.2%	0.2%	0.3%	0.2%	0.1%
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__;f__;g__	0	0.3%	0.8%	0.3%	0.1%	0.1%
k__Bacteria;p__Verrucomicrobia;c__TP21;o__;f__;g__	0	0.0%	0.1%	0.0%	0.0%	0.0%

Relative abundance of OTU taxanomic assignment for treatments based on cDNA template.



Legend	Taxonomy	Total count	Total %	Control %	Low %	Mid %	High %
	No blast hit;Other;Other;Other;Other	0	0.4%	0.5%	0.4%	0.4%	0.2%
	k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__WCHD3-02;g__	0	0.1%	0.2%	0.1%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Armatimonadetes;c__SJA-176;o__;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__;g__	0	9.2%	13.4%	11.2%	9.7%	2.3%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__	0	0.5%	0.0%	0.1%	0.6%	1.2%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Paludibacter	1	13.2%	6.6%	9.3%	15.7%	21.4%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	0	0.0%	0.0%	0.0%	0.0%	0.0%

k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__	0	2.7%	2.9%	2.5%	3.2%	2.1%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	0	12.0%	8.5%	11.0%	21.1%	7.6%
k__Bacteria;p__Chlamydiae;c__Chlamydiae;o__;f__;g__	0	0.1%	0.3%	0.0%	0.0%	0.0%
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__SHD-231	0	0.0%	0.0%	0.0%	0.1%	0.1%
k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__YS2;f__;g__	0	1.1%	0.7%	1.8%	1.0%	1.0%
k__Bacteria;p__Elusimicrobia;c__Elusimicrobia;o__Elusimicrobiales;f__Elusimicrobiaceae;g__	0	0.1%	0.1%	0.3%	0.1%	0.0%
k__Bacteria;p__Elusimicrobia;c__Endomicrobia;o__;f__;g__	0	0.1%	0.1%	0.2%	0.0%	0.0%
k__Bacteria;p__Fibrobacteres;c__Fibrobacteres;o__Fibrobacterales;f__Fibrobacteraceae;g__Fibrobacter	0	2.4%	0.9%	1.6%	4.4%	2.6%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0	0.1%	0.0%	0.1%	0.1%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	0	0.6%	0.6%	0.7%	0.8%	0.5%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Catabacteriaceae;g__	0	0.5%	0.7%	0.4%	0.6%	0.2%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Anaerofustis	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__	0	0.5%	0.3%	0.4%	0.8%	0.5%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Butyrivibrio	0	0.1%	0.1%	0.1%	0.2%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Clostridium	0	0.0%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Coprococcus	0	0.0%	0.0%	0.0%	0.1%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Eubacterium	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Roseburia	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Shuttleworthia	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__	0	1.1%	1.0%	1.0%	1.3%	0.9%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium	0	0.1%	0.1%	0.1%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Eubacterium	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Ruminococcus	0	0.3%	0.3%	0.4%	0.6%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__	0	0.7%	0.6%	0.4%	1.2%	0.6%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Desulfosporomusa	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Propionispora	0	0.4%	0.1%	0.1%	1.1%	0.2%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Selenomonas	2	39.8%	44.6%	40.8%	28.4%	45.3%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Thermosinus	0	1.5%	0.8%	1.4%	1.8%	1.8%
k__Bacteria;p__Lentisphaerae;c__Lentisphaerae;o__Victivallales;f__;g__	0	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Lentisphaerae;c__Lentisphaerae;o__Victivallales;f__Victivallaceae;g__	0	2.4%	5.2%	3.4%	0.5%	0.4%
k__Bacteria;p__Lentisphaerae;c__Lentisphaerae;o__Z20;f__;g__	0	0.6%	1.0%	0.8%	0.4%	0.3%
k__Bacteria;p__Planctomycetes;c__Planctomycea;o__Pirellulales;f__;g__	0	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Planctomycetes;c__Planctomycea;o__Pirellulales;f__Pirellulaceae;g__	0	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Planctomycetes;c__vadinHA49;o__;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__;f__;g__	0	1.2%	0.5%	1.4%	0.5%	2.5%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__;g__	0	0.6%	1.2%	0.5%	0.7%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__;g__CandidatusOdyssella	0	0.1%	0.0%	0.1%	0.1%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingobium	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Brachymonas	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Delftia	0	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Eikenella	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__;f__;g__	0	0.1%	0.1%	0.1%	0.0%	0.2%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfuromonadales;f__Geobacteraceae;g__Geobacter	0	0.1%	0.0%	0.1%	0.1%	0.0%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Polyangiaceae;g__	0	0.5%	0.1%	2.1%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__	0	0.1%	0.1%	0.1%	0.0%	0.2%
k__Bacteria;p__Spirochaetes;c__M1NP2-04;o__;f__;g__	0	0.1%	0.3%	0.1%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Sphaerochaetales;f__Sphaerochaetaceae;g__Sphaerochaeta	0	0.4%	0.4%	0.4%	0.2%	0.5%
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Spirochaeta	0	0.2%	0.6%	0.4%	0.0%	0.0%
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema	0	2.9%	2.8%	1.9%	1.0%	6.0%
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__TG5	0	0.7%	0.9%	1.7%	0.2%	0.1%
k__Bacteria;p__TM7;c__TM7-3;o__CW040;f__F16;g__	0	0.1%	0.1%	0.1%	0.1%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__Bulleidia	0	0.0%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__L7A_E11	0	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__p-75-a5	0	0.0%	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__vadinHA31;g__RFN20	0	1.2%	1.5%	1.1%	1.6%	0.5%
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae;g__Anaeroplasma	0	0.5%	0.4%	0.5%	0.8%	0.2%
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39;f__;g__	0	0.1%	0.0%	0.1%	0.1%	0.0%
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__;f__;g__	0	0.4%	1.0%	0.3%	0.2%	0.1%
k__Bacteria;p__Verrucomicrobia;c__TP21;o__;f__;g__	0	0.1%	0.2%	0.1%	0.0%	0.0%
k__Bacteria;p__Verrucomicrobia;c__Verruco-5;o__RFP12;f__;g__	0	0.0%	0.0%	0.0%	0.0%	0.0%

OTU table sorted for ANOVA probability (<0.05) with values for treatments indicated as the percentage contribution for the gDNA microbiome

OTU	prob	FDR_corrected	Control_mean	Low_mean	Mid_mean	High_mean	Consensus Lineage
717	7.03E-07	0.001249839	0	0	0	0.001318498	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2495	6.07E-06	0.005393491	0	0	0	0.00058615	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
242	6.25E-05	0.037054026	0.005424599	0.000221386	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
1159	0.000144375	0.064174573	0	0	0	0.000659008	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1162	0.000240383	0.08548002	0.000660301	0.00022234	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
386	0.000310003	0.091864125	0.002346737	0.000148134	7.34E-05	0.000292351	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
505	0.000923897	0.234669781	0	7.43E-05	0.000731238	0.000219493	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
193	0.001291	0.286924797	0.000366381	0.000147591	0.000513192	0.003589083	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
804	0.001906491	0.376637864	0	0	0.000365129	0.000878984	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
1880	0.002125511	0.377915907	0.001099321	0.000519972	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
519	0.00270962	0.437973068	0.000587479	7.38E-05	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
3287	0.004599548	0.681499715	0.000807216	0.000148412	0	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
1037	0.00505421	0.691260357	0.003003678	0.001038035	0.001756031	0.000220313	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
257	0.005140283	0.652815893	0.000732124	0.000963022	7.30E-05	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
1030	0.005153674	0.610882127	0.000220736	0	0.00021946	0.005052708	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
687	0.005295871	0.588503637	0.000732305	7.38E-05	0.000146344	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
2584	0.005635157	0.58937114	0	0	0	0.000806609	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
1417	0.005708986	0.563920949	0.000953314	0.004372017	0.002122495	0.000293508	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
897	0.005753769	0.538431618	0.000292559	7.42E-05	0.000804289	0.000732977	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__; s__
1437	0.005951512	0.529089395	0	0	0.000512084	7.30E-05	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
888	0.00650024	0.550353619	0.000293286	0.00059227	7.31E-05	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
564	0.007690397	0.621523871	0.000439292	7.38E-05	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
1573	0.007925848	0.612702478	0.000439565	7.42E-05	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__; s__
3445	0.00839314	0.621791772	0.000147006	0.000221386	0.000292383	0.002050754	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__

1505	0.009690464	0.6891858	0.000659029	7.43E-05	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
166	0.00984047	0.67293677	0.0036707	0.00170479	0.001315649	0.000440143	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
1566	0.011422205	0.752173356	0.001171779	0	7.34E-05	7.33E-05	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
684	0.011641823	0.739255738	0.00029374	0.000148134	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Z20; f__; g__; s__
666	0.011954069	0.732908091	0.000293013	7.43E-05	0	0	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__; f__; g__; s__
1776	0.011960486	0.708858163	0	7.38E-05	0.000292383	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
1116	0.01226623	0.703527661	7.37E-05	0	0.000292752	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
853	0.012456841	0.692133217	0	0.000962876	0.000219154	0	k__Bacteria; p__Fibrobacteres; c__Fibrobacteres; o__Fibrobacterales; f__Fibrobacteraceae; g__Fibrobacter; s__Fibrobactersuccinogenes
429	0.013756323	0.741174028	0.00022119	0.000148412	0.000293122	0.010700681	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
656	0.013824807	0.722956099	0.000733213	0	7.34E-05	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1387	0.014236063	0.723191977	0	0	0	0.001684091	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
690	0.014613711	0.721754963	0.00014746	0	0.000219332	0.000806655	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
381	0.014762698	0.709407499	0.000219829	0.001852513	0.000291949	0.000657996	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
169	0.015220929	0.712179269	0.001393242	0.002151787	0.000292816	0.00021983	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Z20; f__; g__; s__
3170	0.01523199	0.69442253	0.001465245	0	0.000146408	0.000219685	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1713	0.015448067	0.686666585	7.37E-05	0	7.34E-05	0.000366657	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
79	0.015514607	0.672804172	0.012372641	0.000296546	0.001608387	0.001098086	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
657	0.015673708	0.663520303	0	0.000222472	0.000878257	0.000292688	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
1750	0.015697143	0.649058609	0.000366108	0.001926692	0.001904755	0.006232734	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
332	0.016404761	0.662901475	0.00058648	7.38E-05	0	0.00021983	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
199	0.016515019	0.652526739	0	0	0.000948964	0.003806453	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1313	0.01670023	0.645500204	0	0	0	0.000365837	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__
2142	0.016705055	0.631948685	0.000294466	0	7.34E-05	0.002123606	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1795	0.017543774	0.649850616	0	0.000371016	0	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
3039	0.017627941	0.63964242	0.000294466	0.001329401	0.001756692	0.00469248	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1258	0.017962768	0.638756037	0.000367016	7.42E-05	0.000146408	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
553	0.018852553	0.657251734	0	0	0.000219332	0.00036632	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
3548	0.019016241	0.650209161	0.000220736	0.000370884	0	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
17	0.019209137	0.64441219	0.004336907	0.003185875	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__

2005	0.019976914	0.657758381	0.000293286	0.00022234	0.000146039	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
387	0.020136205	0.650948581	0.001688615	0.000890856	0	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
241	0.020312654	0.644926765	0.001318696	0.000370473	0.000877888	0.001538666	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__; s__
1332	0.021924033	0.68387597	0.00051275	7.43E-05	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2101	0.022274325	0.68282327	0	0	7.30E-05	0.000513338	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
377	0.023116355	0.696625063	0.000732305	0.001257924	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
2720	0.023126342	0.685310588	0.000514476	7.42E-05	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
3454	0.023401142	0.682085747	0.000733578	0.000814067	7.30E-05	7.33E-05	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
556	0.023585008	0.676357163	7.30E-05	0.000370752	0.000877277	0.000365982	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1299	0.023943974	0.675752152	0	7.42E-05	0	0.0055686	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
516	0.023989826	0.666467365	0	0	0.00021909	0.000439998	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__
195	0.024636023	0.673889988	0.001173778	0.001037214	0.000876602	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
3609	0.024696885	0.66531912	0.00154061	0	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
2336	0.025413168	0.67439721	0.00036647	0.000370884	0.000731543	7.35E-05	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__Prevotellaruminicola
3099	0.026104965	0.682568066	0	0.000890591	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
68	0.027482377	0.708169068	0	0.00118768	0	0	k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Myxococcales; f__Polyangiaceae; g__; s__
1152	0.028683046	0.728549361	0.000439746	0.000296267	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__
13	0.02882579	0.721862739	0.002208723	0.000519031	0.000732282	0.061028973	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
3418	0.031991968	0.790023885	0.00095295	0.000148544	7.30E-05	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
3471	0.032330292	0.787441912	0	0.000593489	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus; s__
123	0.033044552	0.793962342	0.005862538	0.005482616	0.000949028	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
3566	0.034237178	0.811649364	7.37E-05	0.00022234	0	0.000513338	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__; s__
1311	0.035312618	0.826129396	0	0.000148544	0	0.001320046	k__Bacteria; p__Fibrobacteres; c__Fibrobacteres; o__Fibrobacterales; f__Fibrobacteraceae; g__Fibrobacter; s__Fibrobactersuccinogenes
363	0.035797773	0.826603119	0.001172597	0.001336356	0.000218962	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
1364	0.036469144	0.831309456	0.000219283	0.000592548	0	7.35E-05	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
444	0.039034644	0.878526554	0.00014628	7.42E-05	0.000584524	7.30E-05	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
1228	0.039149397	0.870095344	7.37E-05	0.000371016	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
3263	0.039238623	0.861312005	0	7.42E-05	0.000365498	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
140	0.039342111	0.853052119	0.003234679	0.001556232	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__

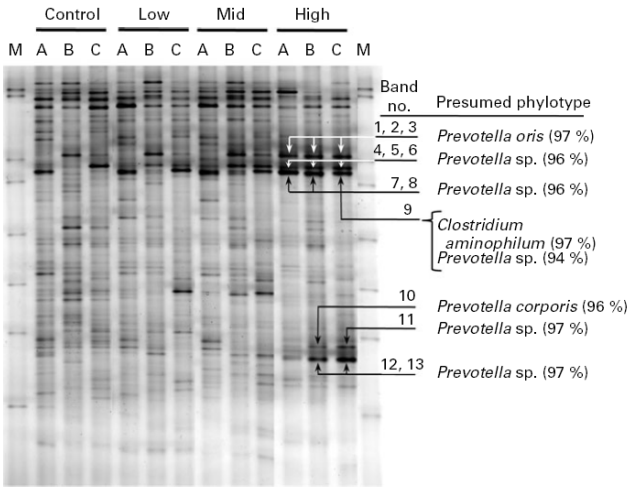
83	0.040305179	0.863404908	0	0	0.000293122	0.000732203	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__
2655	0.042688211	0.903567143	0.0004412	0.001334462	0	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
1742	0.043767167	0.915506145	0	0.000147591	0.000219026	0.000513001	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__Selenomonasruminantium
1841	0.04457068	0.921472902	0.000293286	0	0	0.000219347	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__; s__
1484	0.045334063	0.926482354	0	0.000593489	7.34E-05	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
879	0.045592003	0.921165706	0	0	0.000219154	0.00110007	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
91	0.045948393	0.917935318	0.003302417	0.002075236	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
535	0.046620755	0.921018919	0.000294012	7.42E-05	0.000146408	0.001685248	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2768	0.046996038	0.918230277	0.000146733	0.00022275	0.000584524	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2911	0.047182245	0.911848175	7.30E-05	7.43E-05	7.30E-05	0.000439323	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
686	0.047345433	0.905163218	0.001099686	0.000370619	0.001316068	0.0030079	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__Selenomonasruminantium
1640	0.047921453	0.90642919	7.33E-05	0.000221386	0.000804225	0.000293462	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__
205	0.048089742	0.900037487	0.00249011	0.002524963	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
1178	0.048627731	0.900626107	0.001101139	0.000740271	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__

OTU table sorted for ANOVA probability (<0.05) with values for treatments indicated as the percentage contribution for the cDNA microbiome

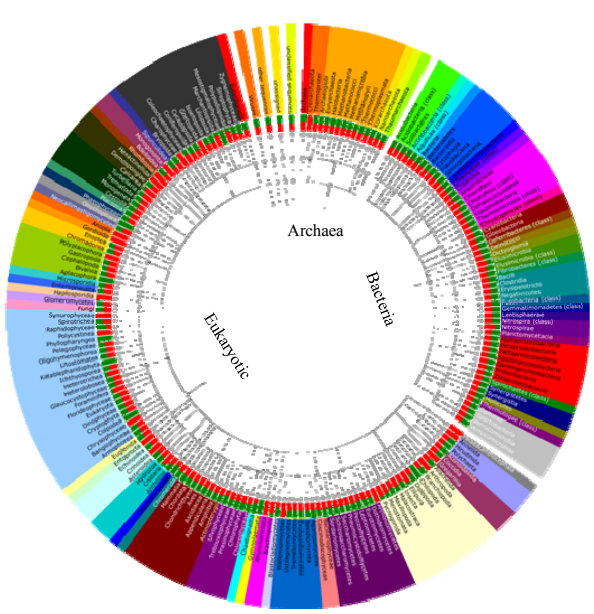
OTU	prob	FDR_corrected	Control_mean	Low_mean	Mid_mean	High_mean	Consensus Lineage
98	2.96E-05	0.052923793	0.000110229	0.000437941	0.002090792	0.00011003	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2163	0.000200087	0.119185153	0	0	0	0.000275247	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
2678	0.000200087	0.178777729	0	0	0	0.000275247	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
943	0.000201018	0.089804637	0	0	0.000275009	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
426	0.000463666	0.165714285	0	5.48E-05	0.001320251	0.000330726	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
2477	0.000538528	0.160391569	0.000550041	5.46E-05	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__; g__; s__
856	0.000551593	0.140813784	0.001209207	0.001586551	0.007259939	0.020044256	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
693	0.000631138	0.140980342	0.000110138	0	0.000770332	0.00011032	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Butyrivibrio; s__
371	0.00095491	0.189602743	0	0	0.000220022	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
3167	0.000987331	0.176436032	0	0	0	0.00022035	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
1178	0.001570895	0.255199025	0.003193608	0.003230911	5.50E-05	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
377	0.001692691	0.252069834	0.000605455	0.000657473	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
1041	0.001703626	0.234183097	0	5.46E-05	0.000275019	0.000825742	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
2180	0.002396048	0.305838439	0.000329745	0	0	0	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__

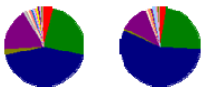
201	0.003354745	0.399661917	0	0.000164276	0	0.000330671	No blast hit
123	0.003861154	0.431242619	0.001593423	0.002080953	0.000164953	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
2809	0.004166928	0.43801767	0.000110229	0.000109272	0.000275009	0.001155577	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
1289	0.004251752	0.422104529	0.000109976	0.000109515	5.50E-05	0.000550458	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
2266	0.006031237	0.567253705	0.000109976	0	0	0.000165199	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
2427	0.006190507	0.553121762	0.000109976	0.000164276	0	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
1394	0.00622329	0.529572383	0.000109976	5.46E-05	0	0.000660216	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
17	0.006760363	0.549125829	0.013656043	0.013854062	0.000384884	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
136	0.006942856	0.539429747	0.000220276	0.000493431	0.001375356	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
997	0.007541503	0.561527734	0	5.46E-05	0.000164944	0.000770591	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
430	0.008427025	0.602363728	0	0.000109757	0	0.000330398	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__
205	0.008514187	0.585186601	0.002252964	0.000109515	0.000109966	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
91	0.008573975	0.567470113	0.003686402	0.003720389	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
644	0.009989154	0.637522106	5.51E-05	0	0.000495213	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
115	0.010980512	0.676626692	0.000165207	0.000383439	0.001980372	5.52E-05	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2343	0.011006429	0.655616275	0.000220114	0.00010964	0	0	k__Bacteria; p__Cyanobacteria; c__4C0d-2; o__YS2; f__; g__; s__
45	0.01127231	0.649794107	0.000880274	0.00103943	0.003631119	0.001157303	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
3583	0.01136184	0.634487779	0	5.48E-05	0	0.000220078	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
1699	0.012117575	0.656185066	5.51E-05	0.000219155	0	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
1200	0.01225222	0.643962252	0	0	0.000109975	0.00099085	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
81	0.013439535	0.686184257	0.000110229	0.000328921	0.000935058	0.002366051	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
598	0.013630296	0.676592746	0.00011032	0	0.000110066	0.000771699	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
709	0.014759072	0.712823289	0	0.000109515	0.000220022	0.000275229	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__; s__
1311	0.016282726	0.765716607	0	0	0.000220113	0.000935227	k__Bacteria; p__Fibrobacteres; c__Fibrobacteres; o__Fibrobacterales; f__Fibrobacteraceae; g__Fibrobacter; s__Fibrobactersuccinogenes
486	0.016863984	0.772716409	0.001156947	0.000821749	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
43	0.017230971	0.76979363	0.016713629	0.009193732	0.003354175	0.002970608	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
2142	0.017473341	0.761581959	0.000110229	0	0.000275291	0.00071593	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1	0.018324271	0.779654111	0.000385088	0.001859227	0.012537263	0.05250312	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
730	0.022343788	0.928566241	0	5.49E-05	0.000385266	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
185	0.022642532	0.919595546	0.00027493	0.000492225	0.000770514	0.003355157	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
2269	0.023478376	0.932352379	0	5.46E-05	0.000275191	0.000550204	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__
239	0.023565625	0.915473309	0.000329999	0.000109515	5.50E-05	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
1880	0.024089443	0.915911366	0.000219769	0.000601974	5.50E-05	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
1030	0.025184998	0.937616489	0.000165298	0	0.000165235	0.001045729	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2795	0.025205534	0.919230398	0.000936052	0.000383674	0.000330097	0.000110302	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
854	0.025475315	0.910487749	0.000220368	0.000492954	0.000109966	0	k__Bacteria; p__Tenericutes; c__Erysipelotrichi; o__Erysipelotrichales; f__Erysipelotrichaceae; g__p-75-a5; s__
326	0.025521741	0.894261787	0.000219607	0.000273556	0.000714781	0.001984025	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
431	0.025602704	0.879846772	0.000220276	0.001367764	0	0	k__Bacteria; p__Elusimicrobia; c__Elusimicrobia; o__Elusimicrobiales; f__Elusimicrobiaceae; g__; s__
1162	0.026577275	0.896105488	0.000934095	0.000328552	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
141	0.02774007	0.917990827	0.00054919	0.00060174	0.000825519	0.005775541	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
3620	0.027756294	0.90182721	0.001703287	0.00120456	0.000384975	0.000165472	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
759	0.027889257	0.88996612	0.00027571	0.000164276	0.000220213	0.00093628	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
241	0.028867884	0.905033477	0.000220276	0	0.000660094	0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__; s__
292	0.029245125	0.901052399	5.51E-05	0.000219272	0.000880588	5.52E-05	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
83	0.030387033	0.92036659	0.000109976	5.46E-05	0.003468428	0.005614394	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__; s__
2358	0.031196772	0.92914387	0	0	0	0.000439883	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
2660	0.03155367	0.924367361	0.000274422	0.001531295	0.00709365	0.027728281	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter;

							s__
79	0.031717156	0.914170281	0.002091418	0.000164636	0.000440444	0.000330925	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
360	0.033883076	0.96109615	0.000495063	0.000437338	0.000440035	0.003465043	k__Bacteria; p__Spirochaetes; c__Spirochaetes; o__Spirochaetales; f__Spirochaetaceae; g__Treponema; s__
1502	0.035433185	0.989360962	0.000164447	0.000164159	0	0.00060619	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
1864	0.035443476	0.974422947	0.000440319	0.000219155	0.000220113	5.49E-05	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
120	0.037678455	1.020172708	0.00352167	0.004440429	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
332	0.037887551	1.010523188	0.001156186	0.000492703	0.000164953	0.000550204	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
988	0.038762475	1.018655034	5.51E-05	0	0.000275019	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
661	0.038864516	1.006534629	5.51E-05	0.000109757	0.000440244	5.52E-05	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Roseburia; s__
2846	0.039093065	0.997990091	0	0	5.50E-05	0.000275538	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
3128	0.039322739	0.989714573	0	0.000109523	0	0.000275229	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
78	0.039467422	0.9795595	0.00104541	0.0036162	0.000604806	0	k__Bacteria; p__Synergistetes; c__Synergistia; o__Synergistales; f__Dethiosulfovibrionaceae; g__TG5; s__
13	0.03987877	0.97621045	0.001818485	0.000493674	0.003139086	0.013719758	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
341	0.040126198	0.968993449	0	0	0.000109975	0.00027552	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
62	0.040409545	0.96282476	0.001045085	0.00049308	0.003357345	0.000165508	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
2344	0.040514455	0.952622774	0	0	0.000109966	0.000275538	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
2945	0.040982555	0.95111463	5.48E-05	0.000328795	0.000165044	5.52E-05	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__
632	0.042081196	0.964090981	0.000165207	0	0.000440062	0	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__; s__
140	0.043216166	0.97756062	0.002863954	0.001586994	0.000164962	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
984	0.044109425	0.985294274	5.48E-05	0	0	0.000439865	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
287	0.045032182	0.99348776	0.000220114	5.48E-05	0.000275009	5.52E-05	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__; s__
529	0.045237489	0.985846262	0	0	5.51E-05	0.000771463	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella; s__
1875	0.045737489	0.984733641	0.000109976	0.000164033	0	0.000440465	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Veillonellaceae; g__Selenomonas; s__
447	0.046257252	0.984067978	0.000604421	0.000547464	0.001210576	0.000605627	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__; g__; s__
289	0.046553635	0.978721724	0.00302329	0.000711732	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__
3252	0.048946006	1.017052481	0.000549788	0.001914835	0.001539955	0.004842478	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Paludibacter; s__
428	0.049074493	1.008001377	0.000769284	0.000382954	0	0	k__Bacteria; p__Lentisphaerae; c__Lentisphaerae; o__Victivallales; f__Victivallaceae; g__; s__



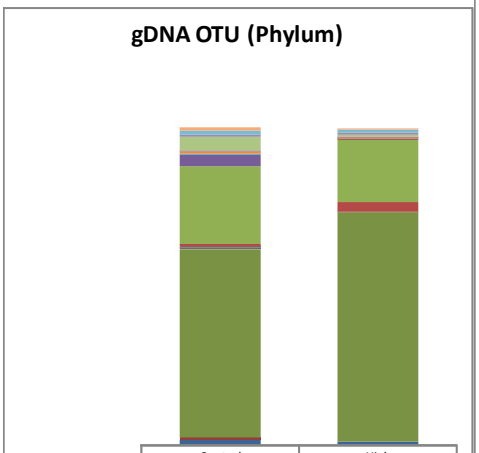
Denaturing gradient gel electrophoresis (DGGE) patterns of rumen microbial community in three individual goats, A, B and C, to which varying dose of bromochloromethane (BCM; low, mid and high) or no BCM (control) were administrated. 16S rRNA gene fragments were amplified from DNA extracted from rumen samples and loaded onto a DGGE gel. Lane M shows markers. The numbered DGGE band indicated by an arrowhead were selected for DNA sequencing.



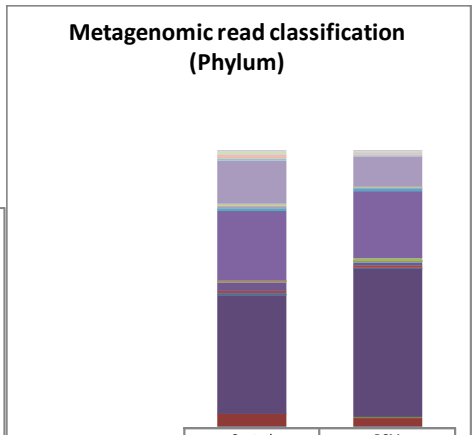


Name	Control	BCM
Actinobacteria	23398 (4.04%)	24375 (3.04%)
Firmicutes	143659 (24.79%)	184880 (23.08%)
Bacteroidetes	247936 (42.79%)	445505 (55.61%)
Cyanobacteria	14087 (2.43%)	9408 (1.17%)
Proteobacteria	101994 (17.60%)	90968 (11.35%)
Verrucomicrobia	5769 (1.00%)	3674 (0.46%)
Tenericutes	1204 (0.21%)	1184 (0.15%)
Deinococcus-Thermus	1253 (0.22%)	1355 (0.17%)
Thermotogae	2340 (0.40%)	2116 (0.26%)
Fusobacteria	4381 (0.76%)	4516 (0.56%)
Nitrospirae	329 (0.06%)	277 (0.03%)
Spirochaetes	5733 (0.99%)	5281 (0.66%)
Lentisphaerae	3910 (0.67%)	1458 (0.18%)
Chloroflexi	3291 (0.57%)	2743 (0.34%)
Synergistetes	3068 (0.53%)	1970 (0.25%)
Planctomycetes	4065 (0.70%)	2589 (0.32%)
Aquificae	1045 (0.18%)	842 (0.11%)
Chlorobi	3478 (0.60%)	3592 (0.45%)
Chlamydiae	1163 (0.20%)	805 (0.10%)
Bacteria	962 (0.17%)	940 (0.12%)
Elusimicrobia	278 (0.05%)	173 (0.02%)
Dictyoglomi	415 (0.07%)	404 (0.05%)
Chrysiogenetes	130 (0.02%)	84 (0.01%)
Gemmatimonadetes	229 (0.04%)	190 (0.02%)
Deferribacteres	576 (0.10%)	518 (0.06%)
Fibrobacteres	2785 (0.48%)	9522 (1.19%)
Acidobacteria	1955 (0.34%)	1780 (0.22%)
Candidatus Poribacteria	17 (0.00%)	16 (0.00%)

This data was calculated for the control and high BCM metagenomes. The data was compared to M5NR using a maximum e-value of 1e-5. Colour shading of the order names indicates phylum membership. Stacked bar charts represent abundance of sequences assigned from control (red) and high BCM (green) metagenomes to the order classification.



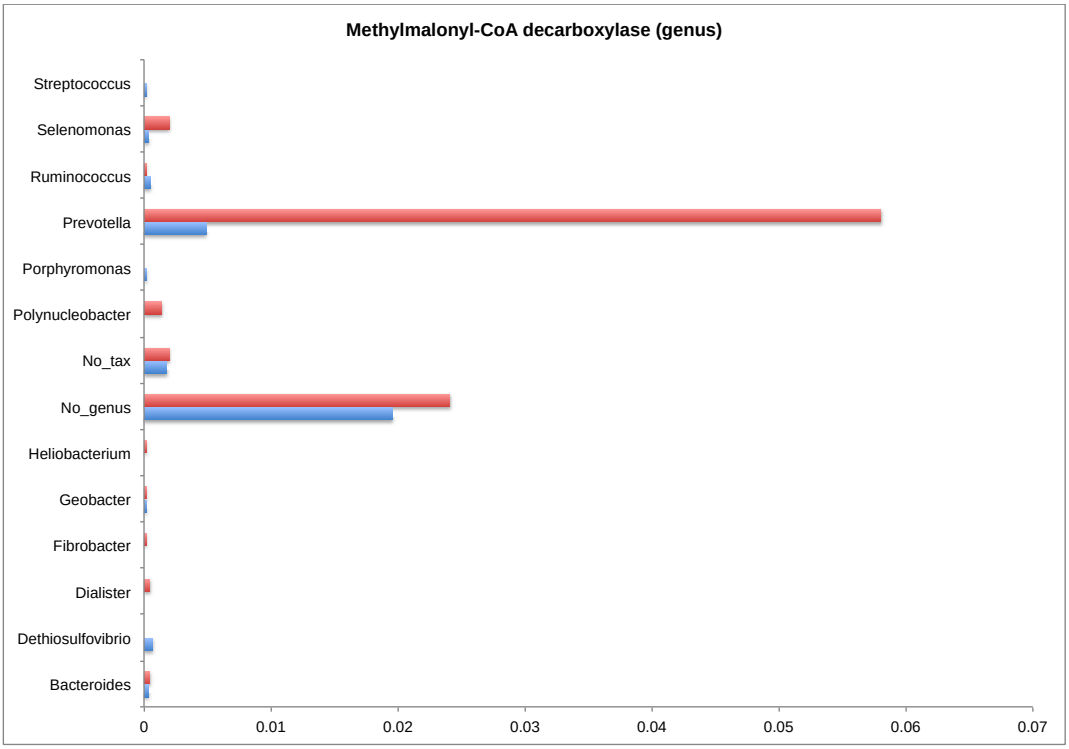
	Control	High
Verrucomicrobia	0.90	0.08
Tenericutes	1.33	0.77
TM7	0.76	0.92
Synergistetes	4.20	0.53
Spirochaetes	0.39	0.34
SR1	0.04	0.02
Proteobacteria	0.80	0.32
Planctomycetes	0.06	0.07
Lentisphaerae	3.89	0.24
Firmicutes	24.41	19.63
Fibrobacteres	0.74	2.95
Elusimicrobia	0.26	0.00
Cyanobacteria	0.34	0.38
Chloroflexi	0.08	0.09
Chlamydiae	0.18	0.00
Bacteroidetes	59.73	72.50
Euryarchaeota	0.58	0.00
No_blast	1.99	1.17

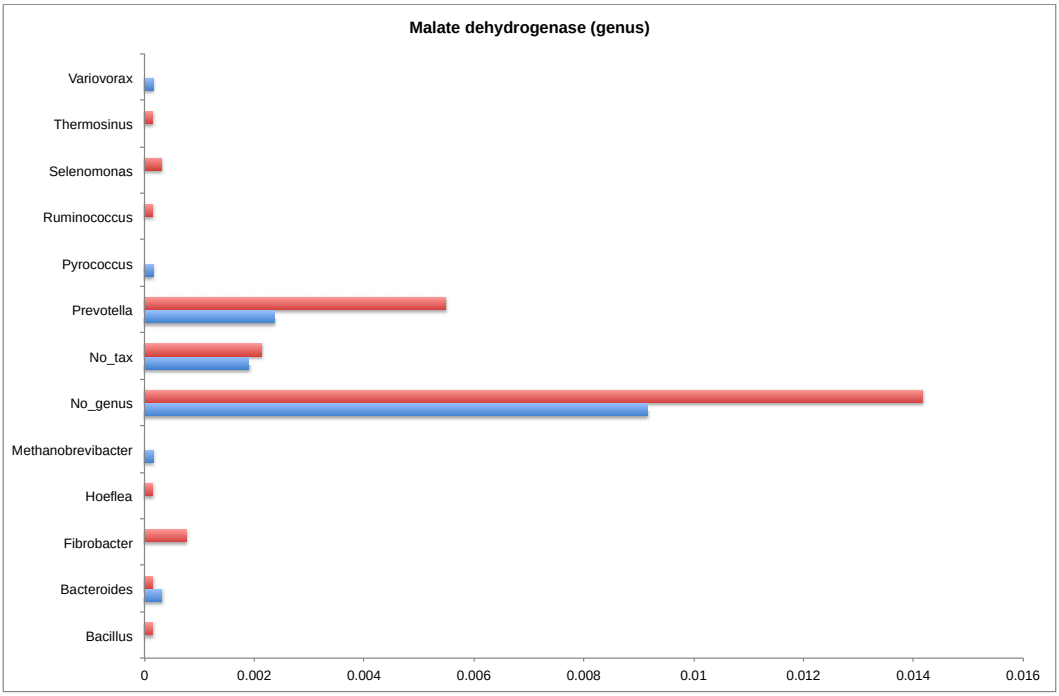


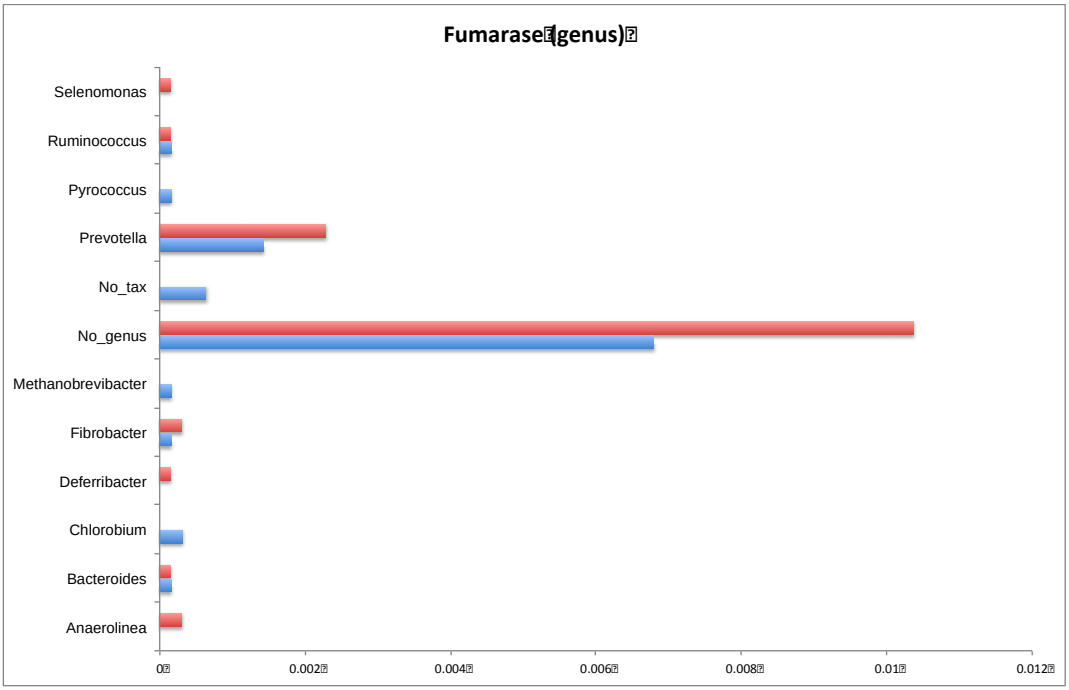
	Control	BCM
unclassified	0.29	0.23
Verrucomicrobia	1.35	0.63
Thermotogae	0.44	0.32
Tenericutes	0.25	0.17
Synergistetes	0.74	0.35
Spirochaetes	0.87	0.61
Proteobacteria	15.41	10.95
Planctomycetes	0.84	0.39
Nitrospirae	0.08	0.05
Lentisphaerae	0.97	0.27
Gemmatimonadetes	0.04	0.03
Fusobacteria	0.82	0.64
Firmicutes	24.70	24.16
Fibrobacteres	0.46	1.12
Elusimicrobia	0.06	0.03
Dictyoglomi	0.08	0.06
Deinococcus-Thermus	0.27	0.20
Deferribacteres	0.12	0.08
Cyanobacteria	2.92	1.46
Chrysiogenetes	0.03	0.02
Chloroflexi	0.68	0.44
Chlorobi	0.68	0.52
Chlamydiae	0.16	0.08
Candidatus Poribacteria	0.01	0.00
Bacteroidetes	42.70	53.49
Aquificae	0.23	0.15
Actinobacteria	4.30	3.20
Acidobacteria	0.51	0.34

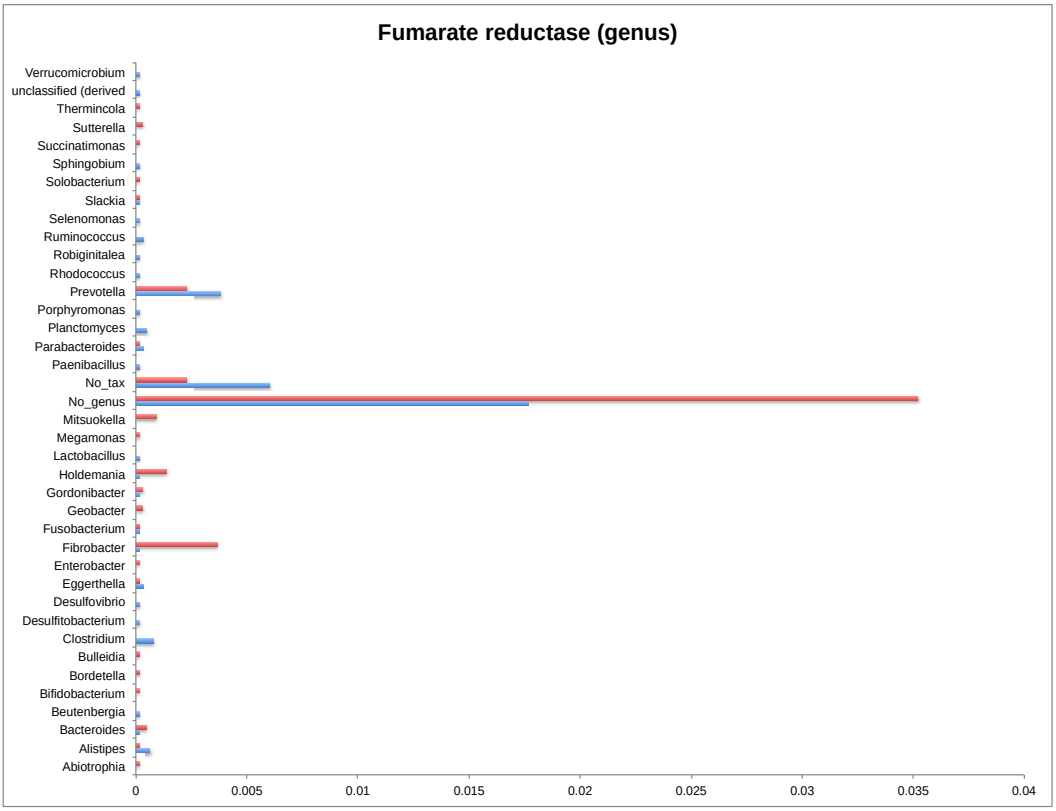
Comparison of OTU assignment for PCR generated amplicons from gDNA using “pyrotags” and classification of metagenomic reads to phlyogentic groups based on “best hit” matches

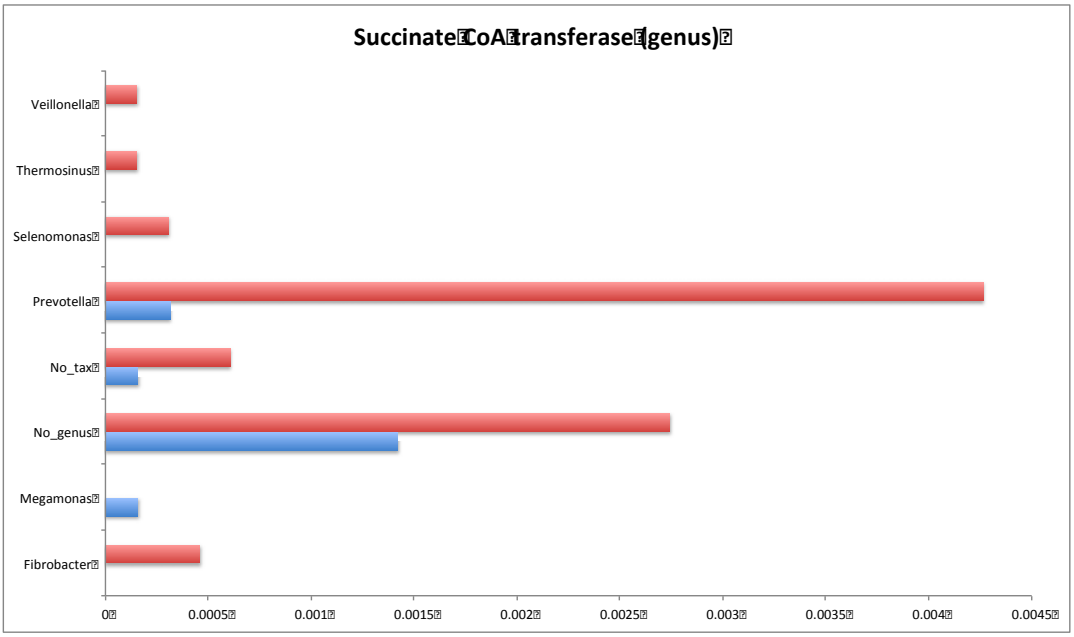
Classification of genes involved in propionate production to the genus level for metagenomic sequences from control (blue) and BCM (red) samples. Values indicate percentage of total metagenomic reads assigned.

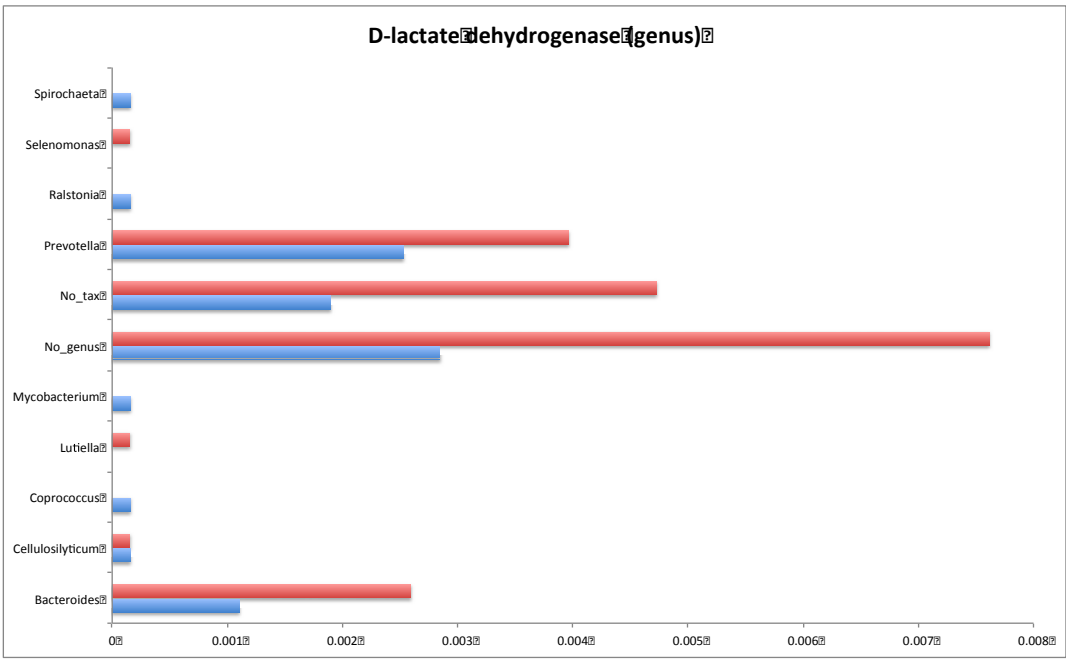


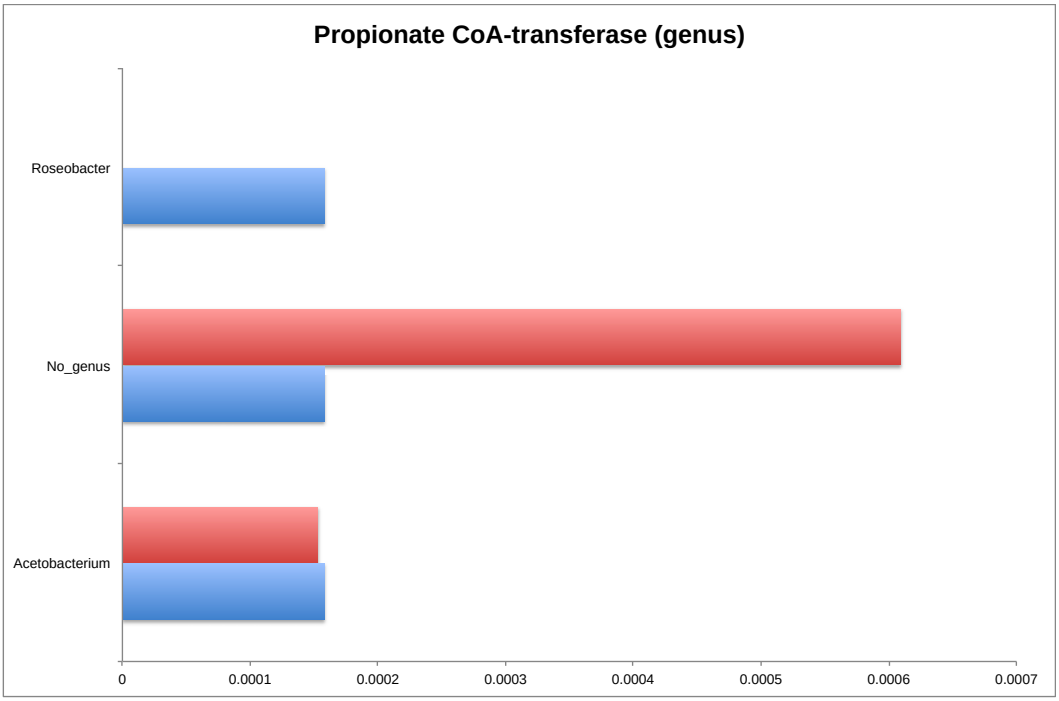

Malate dehydrogenase (genus)
Variovorax
Thermosinus
Selenomonas
Ruminococcus
Pyrococcus
Prevotella
No_tax
No_genus
Methanobrevibacter
Hoeflea
Fibrobacter
Bacteroides
Bacillus
0
0.002
0.004
0.006
0.008
0.01
0.012
0.014
0.016

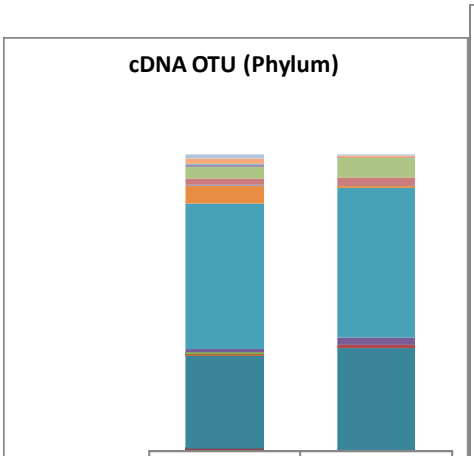

Fumarase (genus)
Selenomonas
Ruminococcus
Pyrococcus
Prevotella
No_tax
No_genus
Methanobrevibacter
Fibrobacter
Deferribacter
Chlorobium
Bacteroides
Anaerolinea
0
0.002
0.004
0.006
0.008
0.01
0.012


Fumarate reductase (genus)
Verrucomicrobium
unclassified (derived
Thermincola
Sutterella
Succinatimonas
Sphingobium
Solobacterium
Slackia
Selenomonas
Ruminococcus
Robiginitalea
Rhodococcus
Prevotella
Porphyromonas
Planctomyces
Parabacteroides
Paenibacillus
No_tax
No_genus
Mitsuokella
Megamonas
Lactobacillus
Holdemania
Gordonibacter
Geobacter
Fusobacterium
Fibrobacter
Enterobacter
Eggerthella
Desulfovibrio
Desulfitobacterium
Clostridium
Bulleidia
Bordetella
Bifidobacterium
Beutenbergia
Bacteroides
Alistipes
Abiotrophia
0
0.005
0.01
0.015
0.02
0.025
0.03
0.035
0.04

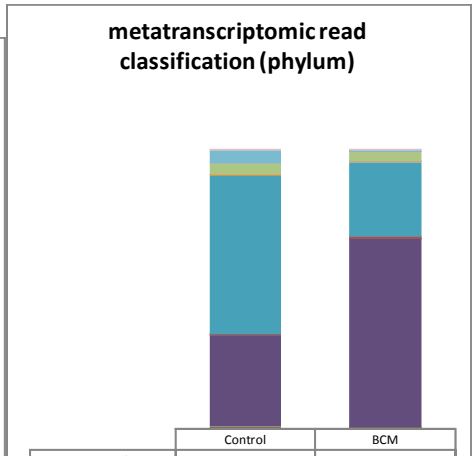

Succinate CoA transferase (genus)
Veillonella
Thermosinus
Selenomonas
Prevotella
No_tax
No_genus
Megamonas
Fibrobacter
0
0.0005
0.001
0.0015
0.002
0.0025
0.003
0.0035
0.004
0.0045


D-lactate dehydrogenase (genus)
Spirochaeta
Selenomonas
Ralstonia
Prevotella
No_tax
No_genus
Mycobacterium
Lutiella
Coprococcus
Cellulosilyticum
Bacteroides
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008


Propionate CoA-transferase (genus)
Roseobacter
No_genus
Acetobacterium
0
0.0001
0.0002
0.0003
0.0004
0.0005
0.0006
0.0007



	Control	High
Verrucomicrobia	1.18	0.10
Tenericutes	1.95	0.83
TM7	0.06	0.01
Synergistetes	0.87	0.09
Spirochaetes	4.05	6.49
Proteobacteria	1.99	3.00
Planctomycetes	0.17	0.03
Lentisphaerae	6.23	0.68
Firmicutes	49.24	50.45
Fibrobacteres	0.87	2.58
Elusimicrobia	0.26	0.00
Cyanobacteria	0.68	0.97
Chloroflexi	0.01	0.06
Chlamydiae	0.25	0.00
Bacteroidetes	31.50	34.55
Armatimonadetes	0.02	0.00
Actinobacteria	0.01	0.01
Euryarchaeota	0.17	0.00
No_blast	0.49	0.17



	Control	BCM
Verrucomicrobia	0.28	0.17
unclassified (derived	0.24	0.27
Thermotogae	0.01	0.05
Synergistetes	4.31	0.45
Spirochaetes	0.32	0.08
Proteobacteria	3.86	3.15
Planctomycetes	0.05	0.17
Lentisphaerae	0.01	0.02
Fusobacteria	0.24	0.20
Firmicutes	56.81	26.59
Fibrobacteres	0.11	0.18
Deinococcus-Thermus	0.03	0.06
Cyanobacteria	0.15	0.50
Chloroflexi	0.06	0.02
Chlorobi	0.16	0.08
Chlamydiae	0.00	0.03
Bacteroidetes	32.62	67.59
Aquificae	0.30	0.01
Actinobacteria	0.37	0.38
Acidobacteria	0.04	0.00

Comparison of OTU assignment for PCR generated amplicons from cDNA using “pyrotags” and classification of metatranscriptomicc reads to phlyogentic groups based on “best hit” matches