Final report

Use of 3-NOP for methane mitigation by programming rumen microbiome development in calves

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Abstract

This project was testing the hypothesis that 3-NOP provided as a feed additive in the pre-ruminant calf will alter the composition of the methanogen and bacterial community, thus reducing methane emissions and improving rumen digestive efficiency which will persist into later life after the inhibitor has been withdrawn. Rumen fermentation profile of calves treated with 3-NOP showed a shift in fermentation from acetate to fatty acids that were longer in length, such as propionate and butyrate, and an increase in formic acid, which is a response to methane inhibition in ruminants. A reduction in methanogen populations and increases of bacterial groups that are involved in H_2 redirection were associated with the treated calves. There were indications of a sustained change to the rumen microbiome at 4 months post-treatment in bacterial groups still being identified as discriminatory species for the previously treated calves. The rumen microbial differences reverted at 52 weeks of age (8 months post-treatment). However, this did not relate to changes in rumen fermentation patterns, or methane production, after the treatment withdrawal. The findings suggest that a greater dose and/or earlier intervention might be required to have a significant lasting effect on methane production later in life.

Executive summary

Background

Technologies that significantly reduce enteric methane emissions from ruminants need to be developed for most grazing operations to reach a carbon neutral position by 2030 (MLA's 'CN30' goal). The company DSM has developed 3-nitrooxypropanol (3-NOP) which acts as a greenhouse gas (GHG) mitigant by inhibiting methane production in the rumen. Recent evidence suggests that changes in colonization of the rumen by microbes prior to weaning may influence the rumen and modify function later in life. The influence of dietary manipulation from birth on growth, methane production, and gastrointestinal microbial ecology in intensively raised dairy calves may be an effective strategy for dairy cow production but needs to be evaluated in extensive beef cattle systems. Novel ways of delivering the compound to grazing cattle under extensive conditions are currently under development at DSM and this proposal aims to demonstrate that 3-NOP, delivered to the calf from birth will influence the rumen microbiome development in a way that reduction in methane will persist for a long period of time after treatment has stopped. This proof of concept must be further evaluated within the Australian red meat sector, as a means of reaching carbon neutrality by 2030.

Objectives

1. Estimate methane reduction by 3-NOP through an early life application strategy for cattle that are grazing native grass/legume pastures in northern Australia.

2. Determine dose response correlation for this early-life application and identify the window of time in which the intervention is most effective during early rumen colonization.

3. Comparison of conventional vs 3-NOP treated calves to show no adverse effect on later animal performance/ animal health and animal welfare parameters, while impacts to animal welfare are unexpected, liveweight gain, behaviour, and appearance will be closely observed through the project.

4. Create knowledge of the underlying rumen microbial structure, function and biological driving forces that characterise the phenotype development of a low methane emitting animal as a consequence of an early-life application of 3-NOP.

5. Evaluate net greenhouse gas mitigation benefit of 3-NOP in extensive grazing systems by this specific application and development of new management practices.

6. Development of a practical application/delivery system for 3-NOP in grazing ruminants in the tropics that specifically targets Early Life microbiome programming and reduces lifetime methane emissions.

7. Apply on farm methane measurement techniques needed for verification of methane mitigating potential of 3-NOP.

We were unable to meet all the planned objectives since supplementation with 3-NOP at the dose used was not effective as expected. No significant differences were detected on methane production, rumen fermentation or body weight at 4 and 8 months after the treatment withdrawal, between the control group and animals that received the treatment early in life. However, there were indications of a sustained change to the rumen microbiome at 4 months post-treatment in

bacterial groups still being identified as discriminatory species for the previously treated calves. The rumen microbial differences reverted at 52 weeks of age (8 months post-treatment).

Methodology

A group of 80 pregnant Bos indicus x Bos taurus cows were selected for the trial. Pregnant cows (n = 80) were further divided into 3 groups. The offspring of each group received one of the following treatments from birth: untreated (placebo treatment) or treatment with 3-NOP resulting in three experimental groups: (a) Control (placebo); (b) low dose (3 mg 3-NOP/kg BW); and (c) high dose (5 mg 3-NOP/kg BW). The high dose treatment group high dose animals were removed from the trial due to unforeseen events not related to the treatment (Appendix 9.3). The calves were offered a creep feed (with or without the inclusion of 3-NOP) from birth until about 14 weeks of age. Rumen fluid and blood samples were collected by stomach tubing and jugular vein respectively, from the calves at 8 ± 2 weeks, 14 ± 2 weeks, 30 weeks ± 2 weeks and 52 weeks ± 2 weeks after birth. Body weight of offspring has been monitored under grazing conditions during the course of the trial. Greenfeed units were used to measure the methane production from the weaners. Two periods of methane measurements (~2 months each) have been performed at 5-6 months and 11-12 months of age.

Results/key findings

The rumen microbial and metabolite profiles from treated calves indicated that animals were consuming the treatment from early in life. Rumen fermentation profile of calves treated with 3-NOP showed a shift in fermentation from acetate to fatty acids that were longer in length, such as propionate and butyrate, and an increase in formic acid, which is a response to methane inhibition in ruminants. A reduction in methanogen populations and increases of bacterial groups that are involved in H_2 redirection, such as the *Succinivibrionaceae* family, *Butyrivibrio sp. Prevotella sp., Rikenellaceae family* (RC9) and other *Lachnospiraceae* were also associated with the treated calves. However, no significant differences were detected on methane production, rumen fermentation or body weight at 4 and 8 months after the treatment withdrawal, between the control group and animals that received the treatment early in life. Interestingly, a sustained change to the rumen microbiome at 4 months post-treatment in bacterial groups was still being identified as discriminatory species for the previously treated calves. The changes were not detected at 52 weeks of age (8 months post-treatment).

Benefits to industry

The objective of the project was that early life intervention could shift the community development into a different (and stable) microbiome with a lower methane emission profile. Unfortunately, we are not able to provide benefits to industry as the objective was not achieved and a lower methane emission profile was not established when the dose tested was withdrawn.

Future research and recommendations

Despite the lack of success in this project, early life programming should be further studied and considered as a strategy for more controlled farming systems, not only as methane abatement strategy but also targeting other desirable phenotypes, such as feed efficiency, health parameters or diet transitioning. We do not rule out the use of this strategy in grazing systems in the future, but a better understanding of the mode of action, window of intervention, dose and new delivery methods should be studied and developed under more controlled conditions before attempting to deploy it in a grazing system. For example, future research might benefit from exploring the impact of directly dosing animals from birth

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1. Background

Technologies that significantly reduce enteric methane emissions from ruminants need to be developed for most grazing operations to reach a carbon neutral position by 2030 (MLA's 'CN30' goal). The company DSM has developed 3-nitrooxypropanol (3-NOP) which acts as a greenhouse gas (GHG) mitigant by inhibiting methane production in the rumen. Recent evidence suggests that changes in colonization of the rumen by microbes prior to weaning may influence the rumen and modify function later in life. The influence of dietary manipulation from birth on growth, methane production, and gastrointestinal microbial ecology in intensively raised dairy calves appears to be an effective strategy for dairy cow production but needs to be evaluated in extensive beef cattle systems. Novel ways of delivering the compound to grazing cattle under extensive conditions are currently under development at DSM and this proposal aims to demonstrate that 3-NOP, delivered to the calf from birth will influence the rumen microbiome development in a way that reduction in methane will persist for a long period of time after treatment has stopped. Encouraging results with 3-NOP have been obtained recently in young cattle by CSIRO. This proof of concept has to be further evaluated within the Australian red meat sector, as a means of reaching carbon neutrality by 2030.

The red meat industry contributes 13.1% of total Australian emissions (Commonwealth of Australia 2017). A total of 72.6% of agricultural emissions are from enteric fermentation by ruminants, and nearly 80% of these emissions arise from beef cattle on pasture. Australia has committed to a 26- 28% reduction in GHG emissions by 2030. Over the past 20 years the red meat sector has achieved substantial reductions in GHG emissions (Wiedemann et al., 2015) but there is increasing pressure from the public and government to further reduce the environmental footprint of red meat. The MLA report "GHG mitigation potential of the Australian red meat production and processing sectors" concluded that large reductions in GHG emissions can be achieved through land management but technologies that significantly reduce enteric methane emissions from grazing animals need to be developed for most grazing operations to reach a carbon neutral position by 2030. This report ranked feed-based additives/inhibitors for grazing cattle as the most promising option to reduce enteric greenhouse gas emissions. Nearly half the national beef cattle herd (~ 24 million) resides in northern Australia, and they typically forage for feed, so the challenge in reducing methane emissions is to develop technologies that can be deployed into this extensive grazing system. DSM's 3-NOP compound the most promising and extensively evaluated inhibitor designed to reduce methane emissions from ruminants. The compound acts as a GHG mitigant by inhibiting the enzyme methyl coenzyme M reductase which is the final step in methane formation by methanogenic archaea organisms (Duin et al., 2016). It will be sold under the trade name 'Bovaer' and has recently been registered in Europe. The efficacy of the compound has been extensively evaluated in dairy and beef feedlot operations but there are few studies in roughage fed cattle (DSM 2019). However our study (Martinez-Fernandez et al., 2018) in Brahman-cross cattle fed a roughage diet, showed that 3-NOP treatment reduced methane output by more than 30% which resulted in a shift of the rumen microbial profile to a greater abundance of proteolytic bacteria and a reduction in amino acid fermenting bacteria as well as a shift to increased propionate production which should result in more efficient nitrogen digestion and energy for the animal. This compound has also been used in lactating dairy cattle and beef feedlot animals where similar shifts in rumen fermentation were demonstrated which resulted in increased live-weight gains and feed conversion efficiency (Hristov et al., 2015, Vyas et al., 2018). Here, we plan to investigate an application that could maximize the emission reduction effect on animals in extensive grazing systems by targeting the methane producing microorganism present in the early life ruminant (calf). Scientific rationale: Acquisition of the intestinal or rumen microbiota begins at birth, and a stable microbial community develops by successive colonisation of the gut with key microorganisms. The microbial population in the rumen is characterized by a highly diverse population of bacterial, protozoal, fungal and archaeal (methanogen) species. Several studies have shown that in young ruminants and during rumen development, ingested microbes colonise and establish in a defined and progressive sequence (Stewart et al., 1988). Methanogenic archaea and cellulolytic

bacteria have been found in the undeveloped rumen well before the ingestion of solid feed begins (2-4 days) and then progressively change over 3-6 months as the rumen develops (Fonty et al., 1987, Morvan et al., 1994). It was first reported that hay or grain rations fed early in life have an impact on the bacterial population that established in the rumen (Eadie et al., 1959). However, these and other studies did not employ molecular methods of microbial detection and work in our laboratory using molecular ecology approaches (Gagen et al., 2012, Abecia et al., 2018) (Mi et al, unpublished) has shown that colonisation proceeds differently from that previously accepted.

Based on detailed molecular biology studies in various mammalian systems (humans as well as animals), it is now recognized that the developing microbiome exhibits a degree of plasticity during microbial colonization of the gut that occurs soon after birth and therefore provides a window of opportunity for targeted manipulation, which ultimately can achieve a potential long-lasting low methane phenotypic outcome later in adult life. Recent research indicates that simple nutritional interventions and/or inoculants applied early in life modify the structure of the bacterial population colonizing the rumen and that the effect persists later into life (Yáñez-Ruiz et al., 2015, Abecia et al., 2013, De Barbieri et al., 2015, Meale et al., 2021). For example, it has been demonstrated that feeding a halogenated aliphatic hydrocarbon compound to goat kids prior to weaning continued to decrease methane for up to three months after weaning despite the additive being removed at weaning (Abecia et al., 2014, Abecia et al., 2013). Thus, there is emerging data that suggests application of additives during the first few weeks of an animal's life influences animal productivity and product quality much later in life. In short: the targeted intervention may shift the community development into a different (and stable) microbiome with a lower methane emission profile. Encouraging results in reducing methane with 3-NOP by Early Life Microbiome Programming have been obtained recently in young cattle by CSIRO.

2. Objectives

1. Estimate methane reduction by 3-NOP through an early life application strategy for cattle that are grazing native grass/legume pastures in northern Australia.

2. Determine dose response correlation for this early-life application and identify the window of time in which the intervention is most effective during early rumen colonization.

3. Comparison of conventional vs 3-NOP treated calves to show no adverse effect on later animal performance/ animal health and animal welfare parameters, while impacts to animal welfare are unexpected, liveweight gain, behaviour, and appearance will be closely observed through the project.

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5. Evaluate net greenhouse gas mitigation benefit of 3-NOP in extensive grazing systems by this specific application and development of new management practices.

6. Development of a practical application/delivery system for 3-NOP in grazing ruminants in the tropics that specifically targets Early Life microbiome programming and reduces lifetime methane emissions.

7. Apply on farm methane measurement techniques needed for verification of methane mitigating potential of 3-NOP.

3. Methodology

3.1Animal ethics approval

Animal ethic approval certificates have been obtained from the CSIRO animal ethics committee for the purchase of pregnant cows (ARA 21-01) and for the supplement trial with cows and calves (ARA 21-08).

3.2Experimental protocol:

The project is being conducted at CSIRO Lansdown Research Station near Townsville. A group of 80 pregnant *Bos indicus* x *Bos taurus* cows and offspring have been used during the trial (Animal ethics approved please see Appendix 1). Pregnant cows (n = 80) have been further divided into 3 groups (the 3 groups had similar spread in pregnancy stages to allow similar calving periods, allocation was based on the preg-test data already collected from the cows and are held in three separate yards (~ D0.75 hectare). The offspring of each group received one of the following treatments from birth: untreated (placebo treatment) or treatment with 3-NOP (product labelled as CLOU-3) resulting in three experimental groups: (a) Control (placebo); (b) low dose (3 mg 3-NOP/kg BW); and (c) high dose (5 mg 3-NOP/kg BW). The treatments were only offered to the calves. The high dose treatment group high dose animals were removed from the trial due to unforeseen events not related to the treatment (Appendix 9.3).

Table 1. Summary of experimental treatments

Protocol	Treatment	Number
Control	Placebo	24
Low dose	3 mg 3-NOP/kg BW Equivalent to 30 mg CLOU-3/Kg BW	22
High dose	5 mg 3-NOP/kg BW Equivalent to 50 mg CLOU-3 / Kg BW	24

The calves were offered a creep feed (with or without the inclusion of 3-NOP) from birth until about 14 weeks of age. The dams did not have access to the creep feeders and will be removed from the study after the calves are weaned. Rumen fluid and blood samples have been collected by stomach tubing and jugular vein respectively, from the calves at 8 ± 2 weeks, 14 ± 2 weeks and 30 weeks ± 2 weeks. The last sampling will be completed during November: 52 weeks ± 2 weeks after birth. Body weight of offspring has been monitored under grazing conditions during the trial.

3.3 Antimethanogen formulation and creep feed preparation

The treatments (Placebo or 3-NOP) were mixed with steam flake barley and molasses, concentration was adjusted through the trial based on calves' intakes and size (Table 2 & Appendix 8.2)

Table 2. Ingredient concentration of creep feed for calves

Ingredients concentration (% wet weight)
89.3% steam flaked barley
6.7-8.7% molasses
4.0-2.0% CLOU-3 or Placebo*

*Adjusted through the trial based on body size and consumption of the calves

The three ingredients were mixed (in house) for four minutes using a feed mixer (mixer coefficient of variance 8%). The treatment mixes (Placebo or CLOU-3 + Barley + Molasses) were prepared separately every 10 days and stored in a cold room at 6 °C until offered to the animals. CLOU-3 was provided by DSM Nutritional Products which confirmed a 10% concentration of 3-NOP. CLOU-3 bag was kept in aluminium sealed bag at 6 °C.

Stability was tested prior to the trial and shown not significant losses when stored at 6 °C in sealed containers for maximum period of 10 days. In addition, samples for stability analyses were collected periodically during the trial and stored at -20°C. The samples have been analysed and confirmed the target concentration of 3-NOP in the feed mix and the absence of 3-NOP in the Placebo mix.

3.4 Supplement intake and calves monitoring

Creep feed was offered to the calves from birth. Refusals were recorded and cleaned daily, with new feeds offered twice a day (morning and afternoon). Staff monitored the calves visits through visual inspections and cameras. It was observed that calves started to eat the creep feed around 2 weeks of age. An estimation of feed intakes and mg of compound per live weight is shown in Appendix 9.2.

3.5 Chemical analysis

Concentrations of volatile fatty acids (VFAs) FAs (acetate, propionate, n-butyrate, iso-butyrate, iso-valerate and n-valerate) were measured by gas chromatography (GC) as described by Gagen et al. (2014). Iso-valerate (3-methyl butyrate) includes 2-methyl butyrate, which co-eluted.

The NH_3-N concentration was determined by a colorimetric method following Chaney and Marbach (1962).

An UltiMate® 3000 HPLC system (Dionex, Sunnyvale, CA, USA) with a dedicated Photodiode Array Detector and an Autosampler was used to determine the presence of organic acids in rumen samples supernatants as described by Gagen et al., (2014).

3.6 Methane measurements

Greenfeed units were used to measure the methane production from the weaners. Two periods of methane measurements (~2 months each) have been performed at 6-7 months and 11-12 months of age.

The Greenfeed Emission Monitors (GEMs) (C-Lock Inc., Rapid City, SD, USA) were placed in the paddock adjacent to water, to measure daily enteric methane emissions. To control the number and duration of methane measurements, pellets were provided by each GEM to each animal in up to a maximum of 4 feeding sessions/d with a minimum of 4 h between sessions.
For emission data to be accepted animals are required to have their head in the unit for at least 2 minutes as detected by a proximity sensor. 20 accepted visits per animal was the minimum requirement for the data to be included in the analysis. Sixty-eight percent of the animals met the minimum requirements for incorporating the data into the analysis. The percentage obtained was in line with C-Lock (Greenfeed system manufacturer) recommendations and considered acceptable for grazing studies.

3.7 Microbial analysis

16S rDNA profiling of the microbial community (bacteria and archaea) have been performed by Illumina Miseq. Quantitative PCR will be used to determine abundance of specific microbial populations identified through high throughput sequencing as important species.
Samples of rumen fluid collected from the calves (3 h post supplement feeding) were processed for microbial diversity analysis of bacteria and methanogens. DNA extractions from rumen samples were performed as described by Martinez-Fernandez et al. (2016). The 16S rRNA genes were used to characterize the microbial populations in the rumen for both bacteria (v3-v4 region) (Klindworth et al., 2013) and archaea (v6-v8 region) (Watanabe et al., 2004, Skillman et al., 2004) respectively. Each DNA sample was amplified using the specific primers and a unique barcode combination as described by de Carcer et al. (2011). Amplification products were visualized by performing gel electrophoresis. Product quantities were calculated, and an equal molar amount of each target product was pooled. The pooled target products were run in a 1.5% agarose gel and bands were visualized and excised under blue light trans-illumination. The amplicons were gel purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) prior to submission for 2 × 250 bp Illumina MiSeq sequencing.

Paired-end short-read sequence data generated on the Illumina MiSeq was processed using the VSEARCH package (Rognes et al., 2016). De-multiplexed paired-end sequences were passed through cutadapt for primer removal (Martin, 2011) and then merged prior to sequence quality filtering, followed by error correction, (Edgar and Flyvbjerg, 2015) chimera checking, (Edgar et al., 2011) and clustering of sequences to Amplicon sequence variants (ASVs) (Callahan et al., 2017). Taxonomic classification of bacterial ASVs was done using the IDTAXA algorithm implemented in the DECIPHER R package against the SILVA SSU r132 training set (Murali et al., 2018).

Additional analysis of ASVs was performed in the R packages breakaway, Phyloseq, and the ggplot2 graphics package (Willis, 2019; McMurdie and Holmes, 2013; Wickham, 2016). Identification of ASVs contributing to a microbiome signature, characterising treatment groups was performed in R using the mixOmics package (Rohart et al., 2017). A supervised sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was used with centred log ratio transformation of the count data. The number of selected ASV's for each component and number of components to use in the final model was optimised and validated using cross validation (10-fold), repeated 100 times. The lowest prediction error rate for each cross validation was then used to determine the selected ASV features at each component and used as parameters in the final model. Sample plots displaying similarities between samples in a reduced space spanning the first two latent components of the final sPLS-DA model were produced using the calculated component scores. Confidence ellipse on the sample plots are for each treatment group, reflecting the 95% confidence level for a pairwise confidence region.

Selected DNA samples will also be used as templates for quantifying the abundance of the mcrA gene for total methanogens, and the 16S rRNA for *Methanobrevibacter* genus and *Methanomassiliicoccaceae* family. The primers and assay conditions used will be as previously published by Denman et al., (2007) and Huang et al., (2016). Changes in targeted populations will be calculated using a relative quantification calculation and the 2−ΔΔCt method, with the control group used as the calibrator and total bacterial ct (cycle threshold) values used as the reference value W.

3.8 Metabolomic analysis

3.8.1 Sample preparation

Rumen fluid and plasma samples were thawed at room temperature and left on ice for sample preparation. For each rumen sample, a total volume of 200 μL was prepared consisting of a 160 μL aliquot of rumen fluid, 20 μL 1.5 M potassium phosphate buffer (pH 7.4), and 20 μL DDD. DDD used was a mix of an internal chemical shift standard 1 mM 2,2-dimethyl-2-sila-3,3,4,4,5,5-hexadeuteropentane-5-sulfonic acid (DSS) and internal pH indicator, 1 mM 1,1-difluoro-1-trimethylsilanyl methyl phosphanic acid (DFTMP) dissolved in D2O. For plasma sample, a total volume of 200 μL for each sample was prepared consisting of a 100 μL aliquot of plasma sample, 80 μL 63.75 mM sodium phosphate buffer (pH 7.4), and 20 μL D2O.

3.8.2 NMR spectra collection

The prepared rumen fluid and plasma samples (200 μL) were transferred to NMR tubes. 1H NMR spectra were acquired using a Bruker AV900 NMR spectrometer operating at a 1H frequency of 900.13 MHz and equipped with a 5 mm self-shielded z-gradient triple resonance probe.
The rumen fluid 1H NMR spectra were collected at 298 K. For all rumen samples, a 1D NOESY spectrum was acquired with the standard noesypr1d pulse sequence [(RD)−90o−$t1$−90o−τm−90o−acq]. The water signal was suppressed by continuous wave irradiation during both the relaxation delay of 4.0 s, and the mixing time (τm) of 100 ms. For rumen ID NOESY spectra 131072 data points with a spectral width of 20 ppm were collected into 128 transients and 8 dummy scans. Additionally, 1H–13C heteronuclear single quantum coherence (HSQC) spectra were measured for one of the pooled quality controls (PQCs) of the rumen fluid samples and used as an aid in metabolite identification.
The plasma 1H NMR spectra were collected at 310 K. 1D Carr-Purcell-Meiboom-Gill (CPMG) spectra were acquired with the standard cpmgpr1d pulse sequence [RD−90o−(τ−180o−τ)n−acq]. A fixed spin-spin relaxation delay 2nτ of 80 ms (τ=500 μs) was used to eliminate the broad signals from high molecular weight analytes, and water suppression irradiation was applied during the relaxation delay of 4.0 s. For plasma 1D CPMG spectra 65536 data points with a spectral width of 20 ppm were collected into 128 transients and 16 dummy scans. 2D J-resolved (J-RES) spectra were also acquired for all plasma samples using the standard jresprqf pulse sequence. Spectral widths of 16 ppm in the direct dimension (F2) and 80 Hz in the indirect dimension (F1) were used. J-RES spectra were used to aid with metabolite identification and were not used for further analysis. As a further aid for metabolite identification, HSQC spectra was measured for one of the PQCs of the plasma samples.

3.8.3 Data processing

Signal processing of 1H NMR spectra was performed in TopSpin version 3.6.2 (Bruker Biospin, Rheinstetten, Germany). The free induction delays (FIDs) of the rumen fluid 1D NOESY spectra were multiplied by an exponential window function with 0.10 Hz line broadening, before Fourier transformation. The rumen fluid 1D NOESY spectra were aligned to DSS signal peak (δ=0 ppm). The FIDs of the plasma 1D CPMG spectra were

aligned to the anomeric proton of glucose (δ=5.223 ppm). Phase and baseline correction were performed manually in TopSpin.
Spectral peak alignment was performed in MatLab (MathWorks, Massachusetts, USA), correcting pH ionic shift variations, using the icoshift algorithm (Savorani et al., 2010). Icoshifted spectra were reduced to consecutive integral regions (buckets) of 0.01 ppm and 0.001 ppm width; subsequent multivariate statistical analysis (MVSA) was performed in SIMCA 16 (Sartorious Stedim AB, Umeå, Sweden).
Bucketed data covered a chemical shift range of δ=10.00−0.25 ppm. To eliminate the effects of imperfect water suppression, both rumen fluid and plasma NMR bucketed spectra excluded the chemical shift region of δ=4.90−4.55 ppm. Additionally, a second round of MVSA was completed for rumen fluid, excluding the acetate chemical shift region δ=1.986−1.833 ppm. Acetate inclusive had the largest effect in the initial MVSA and hindered determination of other significant metabolites. The buckets of each spectrum were normalized to the total intensity of the spectrum to correct for inter-sample differences in weight and dilution.

3.8.4 Multivariate Statistical Analysis (MVSA)

MVSA was performed to reduce the dimensionality of large and complex datasets whilst preserving observed variance (Jolliffe and Cadima, 2016). MVSA was performed in SIMCA 16 (Sartorious Stedim AB, Umeå, Sweden) and used as a means of determining significant metabolites between classification cases and the directionality of significant metabolites. Both unsupervised and supervised methods were used throughout. The MVSA performed on the rumen fluid and plasma datasets included unsupervised analysis in the form of Principal Component Analysis (PCA) and supervised analysis in the form of Partial Least Squares (PLS) and Orthogonal Projection to Latent Structures (OPLS).
Unsupervised PCA was performed as an initial step on the datasets of interest. PCA provides a data summary and is used to show whether data separates, an important step in determining model validity. PCA can provide data simplification which enables a description of biological variation without introducing variation. For models which showed separation in PCA and were supported as valid, supervised analysis was performed. Both PLS and OPLS are biased regression analysis methods, which highlight the causes of separation with a known classification system (e.g., treatment versus control). OPLS is an extension of PLS that acts to remove orthogonal (unrelated) systematic variation. The possibility of introducing bias when using supervised models is the main disadvantage of these analytical techniques. This further contributes to the importance of using PCA to support model validity as well as further model validation (e.g., permutation analysis).

3.8.5 Metabolite identification

Metabolites in both rumen fluid and plasma NMR spectra were identified with Chenomx NMR Suite, version 8.52 (Chenomx Inc., Edmonton, Canada). Potential metabolites were then compared to reference spectra in the bovine metabolome database (Foroutan et al., 2020) and compared to the collected 1D NOESY (rumen fluid) or 1D CPMG (plasma) and HSQC spectra to confirm metabolite assignment.

3.9 Statistical analyses:

Data from calves and weaners have been analysed as a univariate model using the GLM procedure of SPSS (IBM, version 21.0), with the animal as the experimental unit. The effect of treatment has been analysed for rumen fermentation metabolites, methanogens abundances, methane production and body weight. Sex of the animal has been included as random effect for analysing the methane production. Effects will be considered significant at $P \leq 0.05$. When significant differences are detected, differences among means were tested using least significant difference.

4. Results

4.1 Rumen fermentation metabolites, methane production and body weight

The calf treatment (3-NOP) effects on rumen fermentation parameters and body weight are shown in Tables 3-4 (collection 2 & 3). The weaners post treatment (third collection) effects on rumen fermentation parameters, body weight and methane production are shown in Table 6 & 7. No significant treatment effects were observed on body weight during the supplementation period. The trial design ensured that the minimum number of experimental animals was used for measurement of methane production rather than measurements of weight gain.

Table 3. Sex and body weight of calves at birth per experimental group.

	Control	Low dose	SEM	P-value
Number	24	22	-	-
Male	11	10	-	-
Female	13	12	-	-
BW at birth kg	34.3	34.8	0.62	0.699

At 8 weeks of age (Table 4), the major changes in VFAs were a significant increase in butyrate and a decrease in acetate in the low dose calves compared with control group. Regarding the organic acids: citrate, lactate and formate were significantly increased in the low dose calves as well.

Table 4. Rumen fermentation parameters in 8 ± 2 weeks old calves supplemented with placebo

	Control	Low dose	SEM	P-value
BW (kg)	93.0	94.9	1.48	0.534

Rumen pH	6.67	6.52	0.07	0.272
Citrate (mM)	0.43	0.74	0.06	0.019
Succinate (mM)	1.43	2.00	0.59	0.632
Lactate (mM)	0.16	0.32	0.04	0.045
Formate (mM)	1.26	3.24	0.29	0.001
Fumarate (mM)	0.0004	0.0011	0.00	0.248
Ammonia-N (mg /100 mL)	4.12	3.25	0.31	0.164
Total VFA mM	67.8	60.9	2.32	0.143
Individual VFA (%)				
Acetate	65.0	58.6	1.26	0.015
Propionate	22.5	24.0	0.88	0.383
iso-Butyrate	0.92	1.01	0.05	0.373
n-Butyrate	9.21	13.0	0.61	0.003
iso-Valerate	1.21	1.73	0.13	0.054
n-Valerate	1.21	1.44	0.16	0.490
n-Caproate	0.11	0.22	0.04	0.181
ratio A:P	3.12	2.67	0.15	0.139

At 14 weeks of age (Table 5), an increase in propionate (trend) was observed in low dose calves compared with control, while only acetate and iso-butyrate were significantly decreased in that group of animals. Consequently, a significant lower A:P ratio was observed in 3-NOP supplemented calves. On the other hand, butyrate was still significantly increased in the low dose calves. Regarding organic acids, only formic acid was significantly higher in the low dose group.

Table 5. Rumen fermentation parameters in 14 ± 2 weeks old calves supplemented with placebo

	Control	Low dose	SEM	P-value
BW kg	132	134	1.88	0.591

Rumen pH	6.85	7.00	0.04	0.076
Citrate (mM)	0.51	0.48	0.02	0.482
Succinate (mM)	1.29	1.13	0.10	0.423
Lactate (mM)	0.14	0.16	0.02	0.722
Formate (mM)	0.85	2.83	0.36	0.009
Fumarate (mM)	0.001	0.001	0.00	0.928
Ammonia-N (mg /100 mL)	5.16	4.28	0.19	0.028
Total VFA mM	69.2	74.2	1.96	0.211
Individual VFA (%)				
Acetate	70.5	66.8	0.68	0.009
Propionate	17.3	18.3	0.28	0.064
iso-Butyrate	1.08	0.99	0.02	0.032
n-Butyrate	8.94	11.5	0.41	0.004
iso-Valerate	1.40	1.49	0.04	0.339
n-Valerate	0.64	0.72	0.03	0.231
n-Caproate	0.21	0.27	0.02	0.275
ratio A:P	4.11	3.74	0.91	0.045

No significant differences on rumen fermentation metabolites or body weight (Table 6-7) were detected between weaners at 30 weeks of age (4 months after treatment was withdrawn) and 52 weeks of age (8 months after treatment was withdrawn) grazing together at the same paddock.

Table 6. Rumen fermentation parameters in 30 ± 2 weeks old weaners. Previously supplemented with placebo (Control) or 3-NOP (Low dose) treatment (4 months post-treatment).

	Control	Post-treatment	SEM	P-value
BW kg	209.5	208.8	2.78	0.912
				0.912
Rumen pH	7.07	7.18	0.57	0.352
Citrate (mM)	0.23	0.24	0.03	0.802
Succinate (mM)	0.85	0.72	0.06	0.274
Lactate (mM)	0.11	0.08	0.01	0.225
Formate (mM)	0.27	0.25	0.03	0.645
Fumarate (mM)	0.002	0.001	0.00	0.647
Ammonia-N (mg /100 mL)	6.00	5.69	0.20	0.434
Total VFA mM	70.4	66.6	2.04	0.353
Individual VFA (%)				
Acetate	72.6	72.7	0.15	0.620
Propionate	12.36	12.44	0.12	0.713
iso-Butyrate	1.15	1.14	0.02	0.733
n-Butyrate	11.4	11.3	0.10	0.447
iso-Valerate	1.44	1.39	0.03	0.381
n-Valerate	0.79	0.79	0.01	0.933
n-Caproate	0.29	0.27	0.01	0.507
ratio A:P	5.89	5.87	0.06	0.867

Table 7. Rumen fermentation parameters in 52 ± 2 weeks old weaners. Previously supplemented with placebo (Control) or 3-NOP (Low dose) treatment (8 months post-treatment).

	Control	Post-treatment	SEM	P-value
BW kg	249.5	250.6	3.02	0.852
Rumen pH	6.55	6.58	0.58	0.779
Citrate (mM)	0.18	0.19	0.008	0.397
Succinate (mM)	0.49	0.52	0.030	0.554
Lactate (mM)	0.52	0.02	0.006	0.021
Formate (mM)	0.008	0.004	0.002	0.268
Fumarate (mM)	0.0005	0.0004	0.000	0.390
Ammonia-N (mg /100 mL)	6.62	6.37	0.24	0.602
Total VFA mM	85.2	83.0	1.99	0.576
Individual VFA (%)				
Acetate	69.3	69.4	0.13	0.931
Propionate	14.9	14.8	0.14	0.751
iso-Butyrate	1.18	1.26	0.02	0.126
n-Butyrate	12.3	12.1	0.09	0.460
iso-Valerate	1.49	1.58	0.03	0.070
n-Valerate	0.80	0.84	0.01	0.078
ratio A:P	4.66	4.70	0.05	0.700

No significant differences on methane production (g/day or g/kg BW) were detected between weaners at 30 weeks of age (4 months after treatment was withdrawal) or 52 weeks of age (8 months after treatment was withdrawal) while grazing together at the same paddock (Table 8 & 9). Seventy-six percent of the animals

visited the Greenfeeds units at the paddock and it was in line with C-Lock (Greenfeed system manufacturer) recommendations and considered acceptable for grazing studies.

Table 8. Methane production in 30 ± 2 weeks old weaners (2 months period). Previously supplemented with placebo (Control) or 3-NOP (Low dose) treatment (4 months post-treatment).

				P-value		
	Control	Post-treatment	SEM	Post-treatment	Sex	Post-treatment x Sex
Male	9	7	-	-	-	-
Female	7	10	-	-	-	-
Average BW kg	215.7	216.4	3.49	0.717	0.269	0.399
Average methane g/day	120	115	2.22	0.352	0.172	0.625
Average methane g/kg BW	0.56	0.53	0.01	0.490	0.887	0.346

Table 9. Methane production in 52 ± 2 weeks old weaners (2 months period). Previously supplemented with placebo (Control) or 3-NOP (Low dose) treatment (8 months post-treatment).

				P-value		
	Control	Post-treatment	SEM	Post-treatment	Sex	Post-treatment x Sex
Male	9	9	-	-	-	-
Female	7	10	-	-	-	-
Average BW kg	251	253	3.59	0.684	0.186	0.364
Average methane g/day	146	141	2.94	0.401	0.999	0.546
Average methane g/kg BW	0.58	0.56	0.01	0.375	0.415	0.320

4.2 Rumen microbial ecology

Measures of species richness estimates for bacteria indicated lower richness in treated calves (pre-weaning, collection 1: 8 weeks old and collection 2: 14 weeks old) and were considered significantly different for the 14 week old treated calves (Table 10) but this did not affect growth of animals (Table 5). Species richness estimates were not different for calves after the withdrawal of supplement for collections at week 30 and 52.

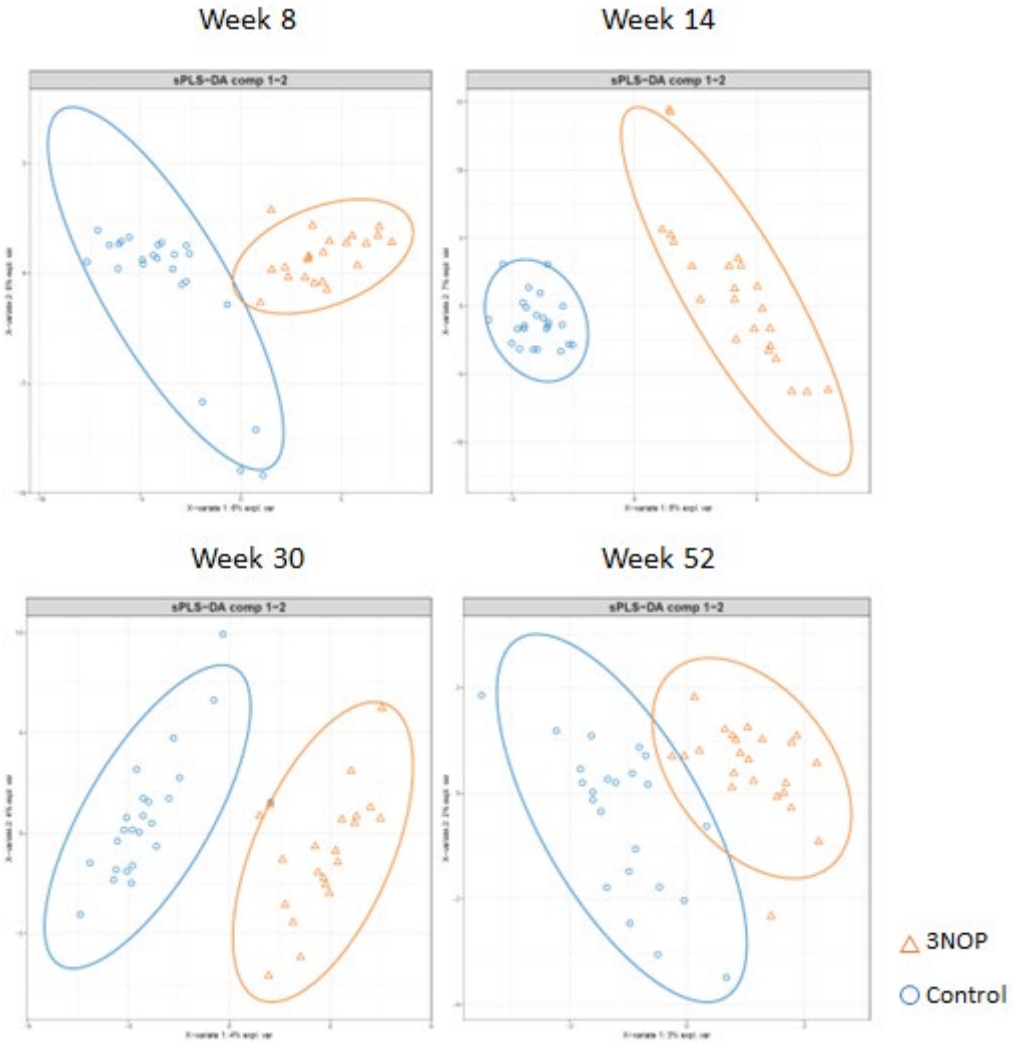
Table 10. Bacterial species richness estimates (breakaway)

	Group	Estimates	SE	p-value
Week 8	Control	4475.216	310.8141	-
	3-NOP	-519.398	445.075	0.243
Week 14	Control	6205.234	256.7349	-
	3-NOP	-1239.57	383.8033	0.001
Week 30	Control	6991.167	318.6522	-
	3-NOP	-273.904	469.3111	0.559
Week 52	Control	5933.236	103.1048	-
	3-NOP	-138.678	140.7308	0.324

When assessing changes to the microbiome using unsupervised principal components analysis (PCA), there were no overall differences in composition, indicating only minor or specific changes to the rumen microbiome when treated with supplement. However, when the most discriminative features in the data were analysed by sparse partial least-squares discriminant analysis (sPLS-DA), clear differentiation in microbial composition between the supplemented and control calves were observed for week 8, 14ad 30 (Fig. 1). Performance testing of the sPLS-DA model for week 52 did not indicate an improvement in the model (data not shown), suggesting no detectable difference between groups. Performance testing for the other collections showed a decrease in the classification error rate, indicating a difference between the treatment groups.

Figure 1. The sample plot for projection onto the first two components of the sPLS-DA model. The supplement treated animals adequately separate on the first component except for week 52

samples.



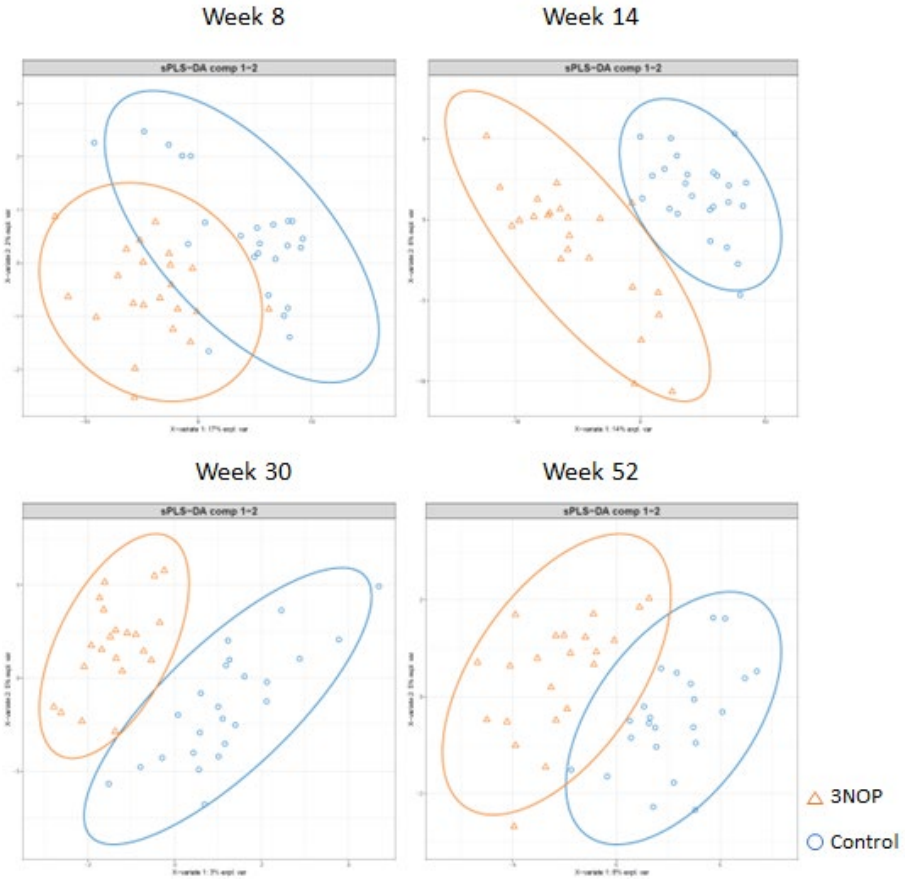
For individual amplified sequence variants (ASVs), the major differences in bacterial genera for the two treatment periods (week 8 and 14) were an increase in relative abundance of species classified within the *Muribaculaceae* (previously S24-7), *Bacteroidales_UCG−001, Bacteroidales_BS11, Succinivibrionaceae*_UCG-002, *Rikenellaceae_RC9*, *Lachnospiraceae, Butyrivibrio, Prevotella, Succiniclasticum* and *Selenomonas* compared to untreated calves. In addition, the relative abundance of *Methanobrevibacter* was decreased in treated calves compared to the controls as a proportion of the bacterial population (Appendix 1). Relative to the controls, the 3-NOP treatment was negatively associated with the abundance of ASVs assigned to *Prevotella*, *Ruminococcus, Christensenellaceae, Desulfovibrio,* and *Bacteroidales_F082*.

The calf microbiome at week 30 after supplement was withdrawn provided evidence of a sustained 3NOP treatment effect on several populations identified from the supplementation period (Week 8 and 14) (Appendix 2). This included *Rikenellaceae_RC9*, *Butyrivibrio, Prevotella, Succiniclasticum* and *Bacteroidales_BS11*. The *Lachnospiraceae* were no longer differentiated from the control and the *Muribaculaceae* species were identified in both the control and previously treated animals.
The poor performance of the final sPLS-DA model for week 52 samples indicates very little changes to the rumen microbiomes that could be attributed to the treatment groups. However, the *Rikenellaceae_RC9* was still associated with the previously 3NOP treated group of calves.

For the archaeal microbiome there was no overall differences in composition using unsupervised principal components analysis (PCA), indicating only minor or specific changes when treated with supplement.

However, when the most discriminative features in the data were analysed by sparse partial least-squares discriminant analysis (sPLS-DA), clear differentiation in microbial composition between the supplemented and control calve groups were observed for week 14 and 30 (Fig. 2). Performance testing of the sPLS-DA model for weeks 8 and 52 did not indicate an improvement in the model (data not shown), suggesting no detectable difference between groups, while all other collections indicated a decrease in the classification error rate, validating changes observed in week 14 and 30.

Figure 2. The sample plot for projection onto the first two components of the sPLS-DA model. The supplement treated animals adequately separate on the first component except for week 8 and 52 samples.



Regarding the archaeal ASVs, the most discriminative ASVs for 3-NOP treatment were an increase in the relative abundance of ASVs classified within *Methanosphaera, Methanobrevibacter oralis* and Methanomassiliicoccaceae group 10 in both supplemented collections (Appendix 3). Archaeal populations that were identified as discriminating between groups, generally were also present in the control group, suggesting the variance detected is at t strain level especially for weeks 30 and 52 (Appendix 4).

4.2.1 Effect on rumen methanogens abundances

Quantitative PCR analysis of the effect of 3-NOP on the abundance of total methanogens (McrA gene), *Methanomassiliicoccaceae* family and *Methanobrevibacter* spp. are shown in Fig. 3-6. The relative abundance *Methanobrevibacter spp*. was decreased 2.5-folds (P = 0.001) compared with the control group when animals were supplemented with 3-NOP at 8 weeks of age (Fig. 3). The total methanogens relative abundance significantly decreased (4.5 folds), while a trend was detected for *Methanomassiliicoccaceae*

family (2-folds decreased) at 14 weeks of age for the treated calves compared to control group. These differences were in the range observed in studies with adult animals.

Non-significant difference was detected between the control animals and experimental group at 30 and 52 weeks of age, 4 and 8 months respectively from withdrawal of supplement.

Figure 3. Quantitative PCR analysis of total methanogens (McrA gene), Methanobrevibacter and Methanomassiliicoccaceae population changes in response to 3-NOP supplementation in 8 ± 2 weeks old calves. The y-axis denotes fold change from control group; * denote significant differences between treatment and control (P ≤ 0.05).

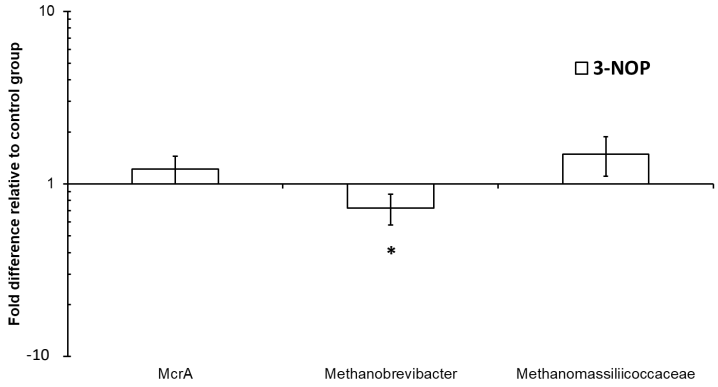


Figure 4. Quantitative PCR analysis of total methanogens (McrA gene), Methanobrevibacter and Methanomassiliicoccaceae population changes in response to 3-NOP supplementation in 14 ± 2 weeks old calves. The y-axis denotes fold change from control group; * denote significant differences between treatment and control (P < 0.001); t denotes a trend between treatment and control (P ≤ 0.1).**

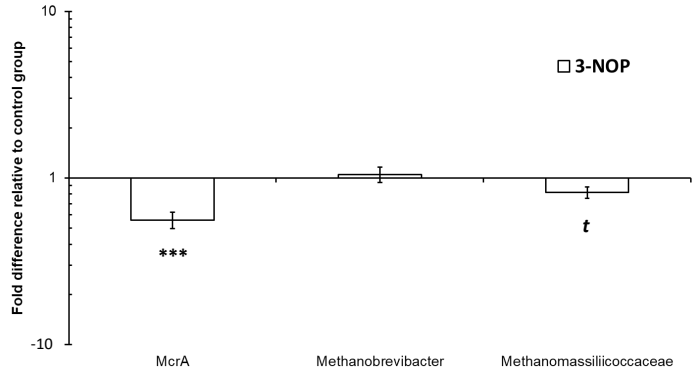


Figure 5. Quantitative PCR analysis of total methanogens (McrA gene), Methanobrevibacter and Methanomassiliicoccaceae population changes in 30 ± 2 weeks old weaners previously supplemented with 3-NOP. The y-axis denotes fold change from control group.

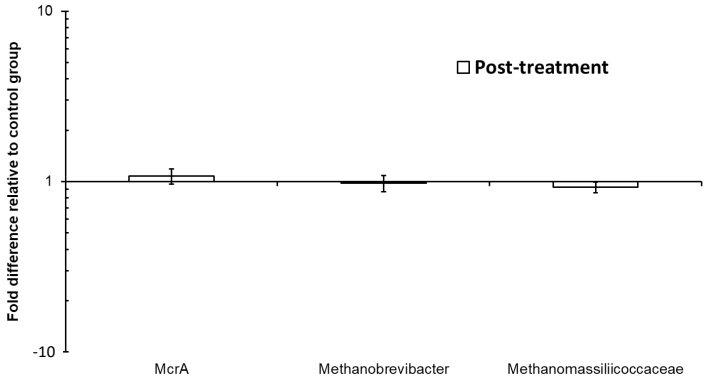
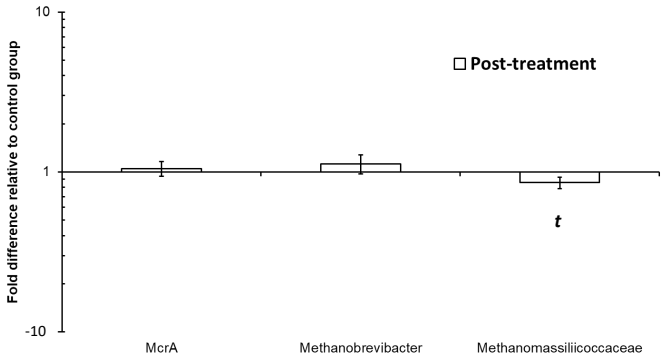


Figure 6. Quantitative PCR analysis of total methanogens (McrA gene), Methanobrevibacter and Methanomassiliicoccaceae population changes in 52 ± 2 weeks old weaners previously supplemented with 3-NOP. The y-axis denotes fold change from control group; t denotes a trend between treatment and control (P ≤ 0.1).



4.3 Rumen and plasma metabolomics

The only differences detected between control and treated calves' ruminal metabolites, coincided with the supplementation of 3-NOP at 14 weeks of age (Fig. 7). Non-significant difference was detected between the control animals and experimental group at 30 weeks of age, 4 months from withdrawal of supplement (Fig. 8).

The rumen metabolites contributing to the differences detected when 3-NOP was supplied to the calves were annotated and shown in Fig. 9. Rumen fluid from treated calves showed an increase in Butyrate, Valerate, alanine, EtOH, N,N-dimethylformamide, succinate, glucose, formate and tyrosine, compared to control group.

Regarding the plasma metabolites, non-significant difference was detected between the control animals and experimental group during the supplementation of 3-NOP (Fig. 10) at 14 weeks of age, and at 30 weeks of age, 4 months from withdrawal of supplement (Fig. 11).

Figure 7. PCA scores plot of calves' rumen fluid during treatment period at 14 weeks of age. Coloring indicates group separation present in PCA. (Legend: yellow is 3-NOP treatment group, red is control group).

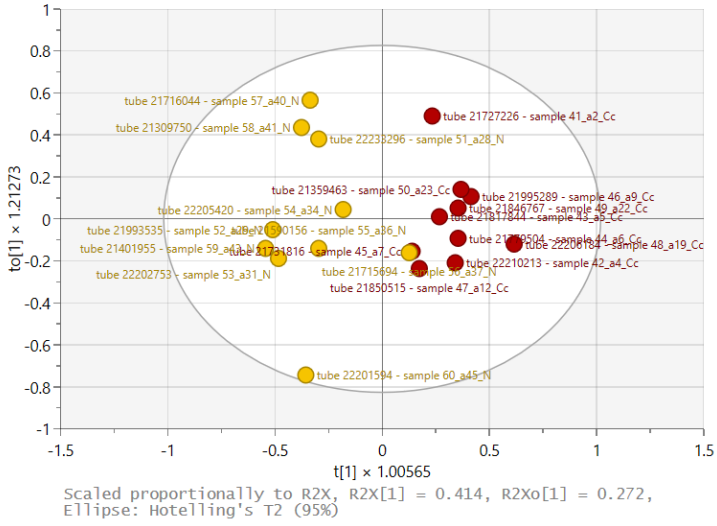


Figure 8. PCA scores plot of calves' rumen fluid during treatment period at 30 weeks of age, 4 months from withdrawal of treatment. Coloring indicates group separation present in PCA. (Legend: cyan is post treatment group, blue is control group).

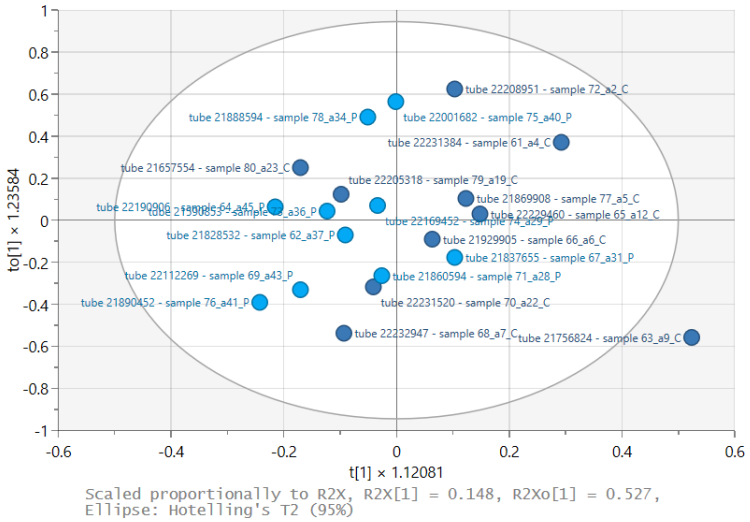


Figure 9. Annotated OPLS 1D S-Plots of the treatment comparison, 3-NOP versus to control, for rumen fluid during treatment period (14 weeks of age). Positive y-axis corresponds with increased levels of metabolite associated with control group and negative y-axis corresponds with increased levels of metabolite associated with 3-NOP treatment.

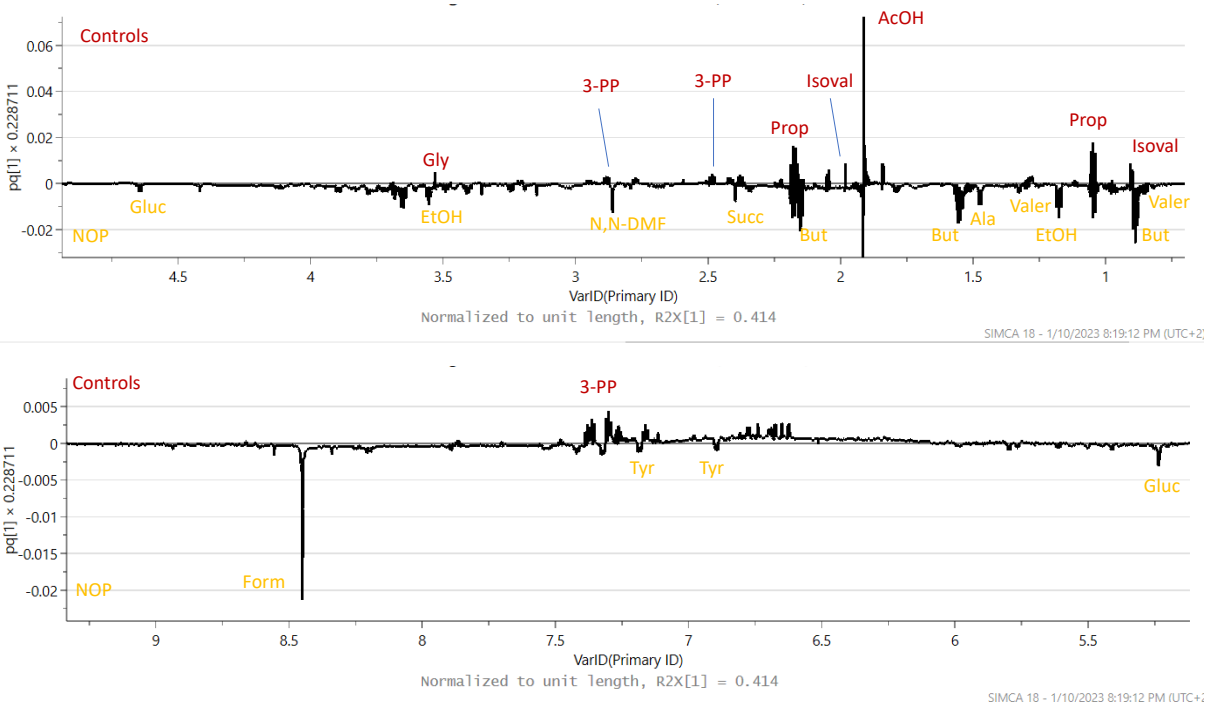


Figure 10. PCA scores plot of calves' plasma during treatment period at 14 weeks of age. Coloring indicates group separation present in PCA. (Legend: red is 3-NOP treatment group, blue is control group).

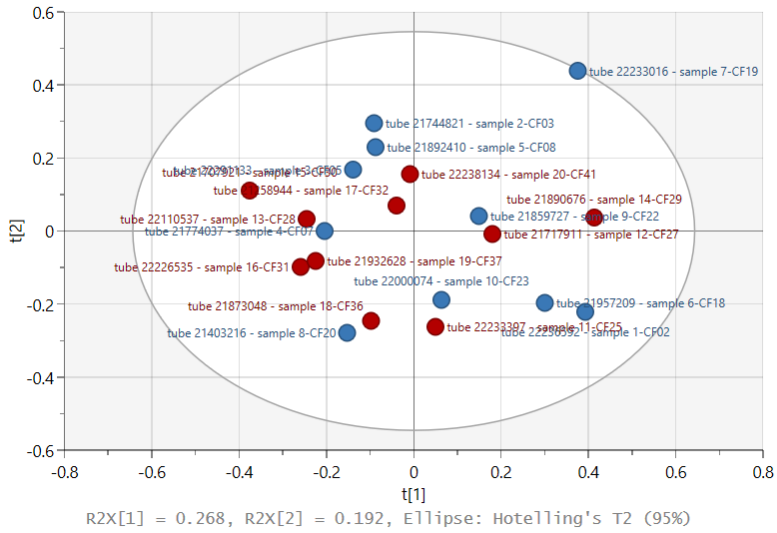
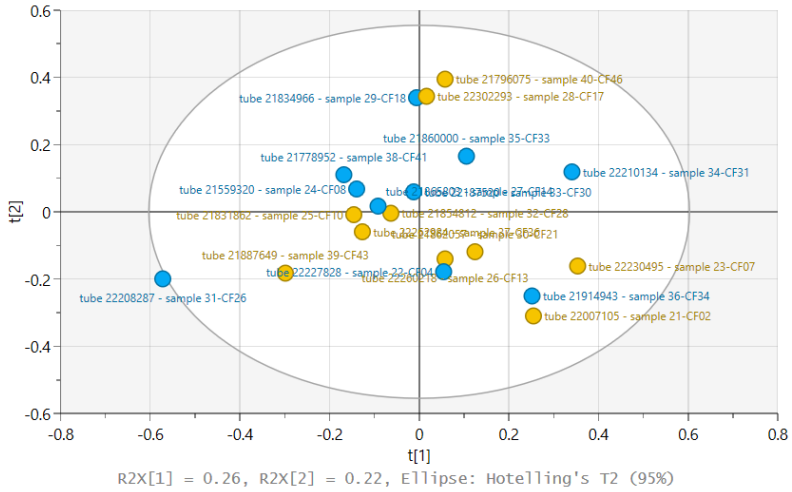


Figure 11. PCA scores plot of calves' plasma during treatment period at 30 weeks of age, 4 months from withdrawal of treatment. Coloring indicates group separation present in PCA. (Legend: cyan is post treatment group, yellow is control group).



5. Discussion

The microbial and metabolite profiles during the treatment period indicate that calves were consuming the supplements from early in life. Rumen fermentation profile of calves treated with 3-NOP showed a shift in fermentation from acetate to fatty acids that were longer in length, particularly propionate that is a major gluconeogenic precursor in ruminants (Newbold et al., 2005) and butyrate. This pattern of fermentation has been reported previously in studies using 3-NOP (Martinez-Fernandez et al., 2014, Haisan et al., 2016, Duin et al., 2016, Haisan et al., 2017, Romero-Perez et al., 2016, Reynolds et al., 2014, Jayanegara et al., 2018) or other methane inhibitors such as halogenated methane analogues (Martinez-Fernandez et al., 2016, Mitsumori et al., 2012, Abecia et al., 2012). Also, increases in formate and other organic acids that might be involved in hydrogen redirection were observed in the low dose calves. A published meta-analysis identified that an increase in formate was a response to methane inhibition in ruminants (Ungerfeld, 2015). and Leng

(2014) has also suggested that formate can accumulate when methanogensis is inhibited, helping to maintain a low partial pressure of H_2 in the rumen fluid.

Rumen microbial changes in calves during the treatment period, also appeared consistent with the rumen metabolic changes observed, mode of action of the compound and elevated hydrogen levels (Martinez-Fernandez et al., 2016, Martinez-Fernandez et al., 2018, Duin et al., 2016). These changes were associated with a reduction in the relative abundance of the methanogens *Methanobrevibacter* (hydrogenotrophic) at 8 weeks of age and *Methanomassiliicoccaceae* (methylotrophic) families at 14 weeks (trend). At a compositional level there was little detectable change to the diversity of methanogen populations, indicating that 3-NOP equally inhibits most rumen methanogenic species.

ASVs classified to bacteria that are involved in H_2 redirection to other metabolic sinks, such as propionate, were associated with 3-NOP treatment. When having access to 3-NOP, mainly the *Succinivibrionaceae* family, *Butyrivibrio* sp. *Prevotella sp., Rikenellaceae* family (RC9) and other *Lachnospiraceae*, were associated to 3-NOP calves. *Succiniclasticum* spp can convert succinate to propionate in the rumen (Van Gylswyk, 1995), and have been previously observed in methanogen inhibited rumen studies. *Succinivibrionacea* species generally are observed in early life prior to transitioning to higher fibre diets and tend to exhibit an inverse relationship to methanogen abundance or inhibition (Furman et al., 2020, Martinez-Fernandez et al., 2016, Pope et al., 2011). Increased butyrate in 3-NOP calves was associated with an increase in *Butyrivibrio* species, which are ubiquitous to the rumen and use a wide range of soluble and some insoluble substrates and characteristically ferment carbohydrates to butyrate (Palevich et al., 2019). The *Prevotellaceae* family has the highest representation of genes for the propionate pathway (Denman et al., 2015) and has been associated with 3-NOP treatment in previous studies (Martinez-Fernandez et al., 2018) and the *Rikenellaceae* family has also been identified to contain enzymes for the succinate to propionate pathway (Denman et al., 2015).

Regarding the post-treatment effect in the weaned animals, no significant differences on methane production, rumen fermentation parameters or body weight were detected between the control and previously treated group of weaners at 4- and 8-months post treatment, when animals were 30 and 52 weeks of age. There were indications of a sustained change to the rumen microbiome at week 30 with *Rikenellaceae_RC9*, *Butyrivibrio, Prevotella, Succiniclasticum* and *Bacteroidales_BS11* still being identified as discriminatory species for the previously treated calves. However, this did not relate to changes in rumen fermentation patterns, likely suggesting that either these alterations are quantitatively small or there has been an alteration in their fermentative function due to changes in the rumen. These findings suggest that programming the rumen early in life might be considered to target other desirable phenotypes (such as feed efficiency, health parameters or diet transitioning) in the adult animal for particular farming systems, if a functional and long-lasting effect can be achieved.

The findings suggest that a greater dose and/or earlier intervention might be required to have a significant lasting effect on methane production later in life, which would make this strategy difficult to implement for grazing systems under current management practices. The target dose (Low dose) used in this study was based on Meale et al., (2021), which showed that calves treated from birth with the antimethanogenic compound 3-nitroxypropanol (3-NOP) showed a different rumen microbial structure and emitted less methane at least a year after treatment ceased. However, in Meale et al., (2021) study animals were dosed orally daily from birth, whereas in current trial supplements were offered to the animals as a group (voluntary intake) to simulate grazing/extensive conditions and minimise the human intervention on calves. It is worth noting that the voluntary intake offering of the supplement might have delayed when animals started consuming the supplement, particularly at early stages of as life as recorded/observed by daily leftover supplement quantification and video recording of the feed trough area where supplement was offered to the calves (Appendix 9.2).

5.1 Communication and extension activities

- An overview of the project was provided by Dr Gonzalo Martinez to attendees at the Livestock Productivity Partnership Field Day held at CSIRO Lansdown Research Station, Townsville on the 7th September 2021.
- CSIRO and DSM discussed a communication strategy for the project, and it was agreed that public promotion of the project or progress reporting to industry and scientific forums would be planned based on the nature of the results as the project progressed. The detail of any public information would be agreed upon between CSIRO, DSM and MLA prior to any public disclosure.
- Chris McSweeney provided an overview of the project to Dairy Australia and it was decided that any application of the technology to the dairy industry should be discussed with Mr Nathan Bird who is the DSM representative in Australia.
- An overview was provided to the Australian Cattle Council board on the 1st November 2022 at Lansdown Research Station. There was significant interest in the approach being taken but no comments about the feasibility of adoption of this practice were made. However, comments about attractiveness of vaccination against methanogens was raised as a management tool and a potential research priority. Also, participants commented that unless the producers can get an increase in productivity when reducing enteric methane, they see adoption quite challenging for methane abatement strategies, due to the cost of the implementation.
- An overview of the project was provided in three separate meetings of GHG Emissions Avoidance Program (EAP) hosted by MLA: 15th December 2022, 10th May 2023 and 14th of November 2023.

6. Conclusion

Results indicate the calves consumed the treatment during the supplementation period and 3-NOP was having the predicted effect on rumen metabolism and microbial community during the pre-weaning period. However, no significant effect was detected on methane production, rumen fermentation metabolites, or body weight after 4 and 8 months of treatment withdrawal. Interestingly, a sustained change to the rumen microbiome was still detected at 4 months post treatment but reverted at 52 weeks of age, which suggest early life intervention could be used as a tool for manipulating and programming the rumen to achieve other desirable phenotypes, if a functional and long-lasting effect can be achieved. It is worth noting that the voluntary intake offering of the supplement might have delayed when animals started consuming the supplement, particularly at early stages of life. The findings suggest that a greater dose and/or earlier intervention might be required to have a significant lasting effect on methane production later in life, which would make this strategy difficult to implement for grazing systems under current management practices.

6.1 Key findings

The rumen microbial and metabolite profiles from treated calves indicated that animals were consuming the treatment from early in life. Rumen fermentation profile of calves treated with 3-NOP showed a shift in fermentation from acetate to fatty acids that were longer in length, such as propionate and butyrate, and an increase in formic acid, which is a response to methane inhibition in ruminants. A reduction in methanogen populations and increases of bacterial groups that are involved in H_2 redirection, such as the *Succinivibrionaceae* family, *Butyrivibrio sp., Prevotella sp. Rikenellaceae_RC9* and other *Lachnospiraceae* were also associated with the treated calves. There were indications of a sustained change to the rumen microbiome at 4 months post treatment with some ASVs still being identified as discriminatory species for

the previously treated calves. However, this did not relate to changes in rumen fermentation patterns or methane production, likely suggesting that either these alterations are quantitatively small or there has been an alteration in their fermentative function due to changes in the rumen.

6.2 Benefits to industry

The project objective was to use early life intervention (using antimethanogenic compound 3-NOP as feed additive from birth) for shifting the community development into a different (and stable) microbiome with a lower methane emission profile once the treatment was withdrawn. Unfortunately, we are not able to provide benefits to industry as the objective was not achieved and a lower methane emission profile was not established when the dose tested was withdrawn. The current results suggest that the practical application of this strategy in an extensive grazing system will be difficult with current management practices and delivery technologies available. Based on published studies, a higher dose or earlier intervention with an antimethanogenic inhibitor might produce a lower methane emission profile, but due to the limitations of extensive grazing practices, the practical application might need to be focussed on more controlled feeding systems (such as dairy industry). Further research will need to be carried out before early life intervention, for targeting a lower methane profile ruminant, might be adopted by the industry.

7. Future research and recommendations

The project was not able to establish a permanent rumen microbiome structure with a lower methane emission profile in the animals that received the antimethogenic inhibitor early in life. In this study the feed supplement with the antimethanogenic inhibitor was offered to the animals as a group (voluntary intake) to minimise the human intervention and simulate extensive grazing practices. It is worth noting that the voluntary intake of the supplement at early stages was a challenge and consumption likely started at a later age that initially targeted. In addition, although the dose tested worked previously (Meale et al., 2021) when calves were dosed orally daily from birth, our findings indicate that a higher dose might be required under grazing management practices.

Despite the lack of success in this project, early life programming should be further studied and considered as a strategy for more controlled farming systems, not only as methane abatement strategy but also targeting other attributes that are desirable in production systems, such as feed efficiency, health parameters or diet transitioning. Further studies, targeting intensive farming systems, would be required to determine the consistency, duration of the response in the maturing animal, optimal route of administration, timing of intervention and the adequate dosage of 3-NOP. We do not rule out the use of this strategy in grazing systems in the future, but a better understanding of the mode of action, window of intervention, dose and delivery method should be studied under more controlled conditions before attempting to deploy it in a grazing system.

8. References

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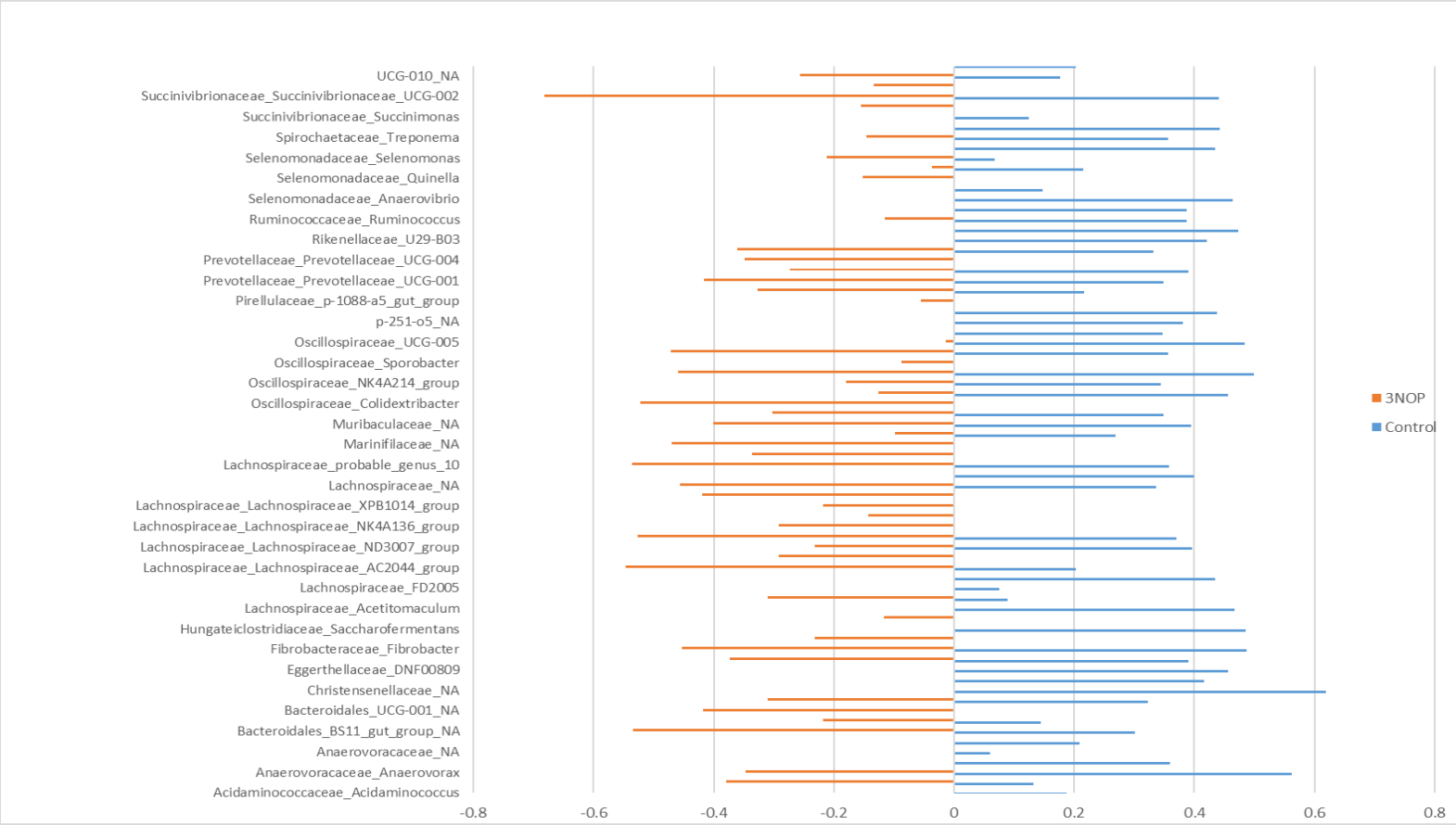
9. Appendix

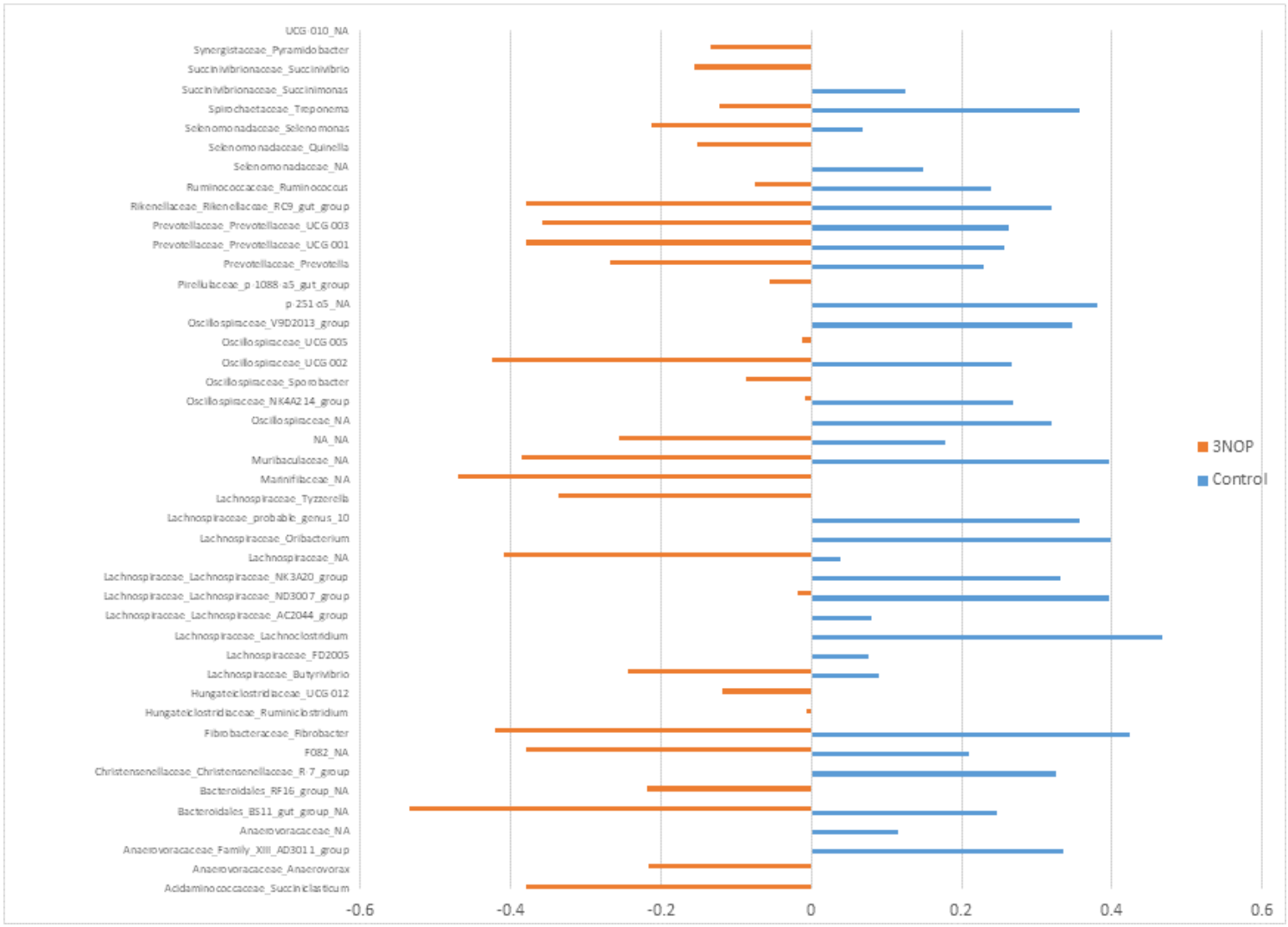
9.1 Microbial ASVs

Bacterial loadings for the most discriminative ASVs for combined weeks 8 and 14 collections



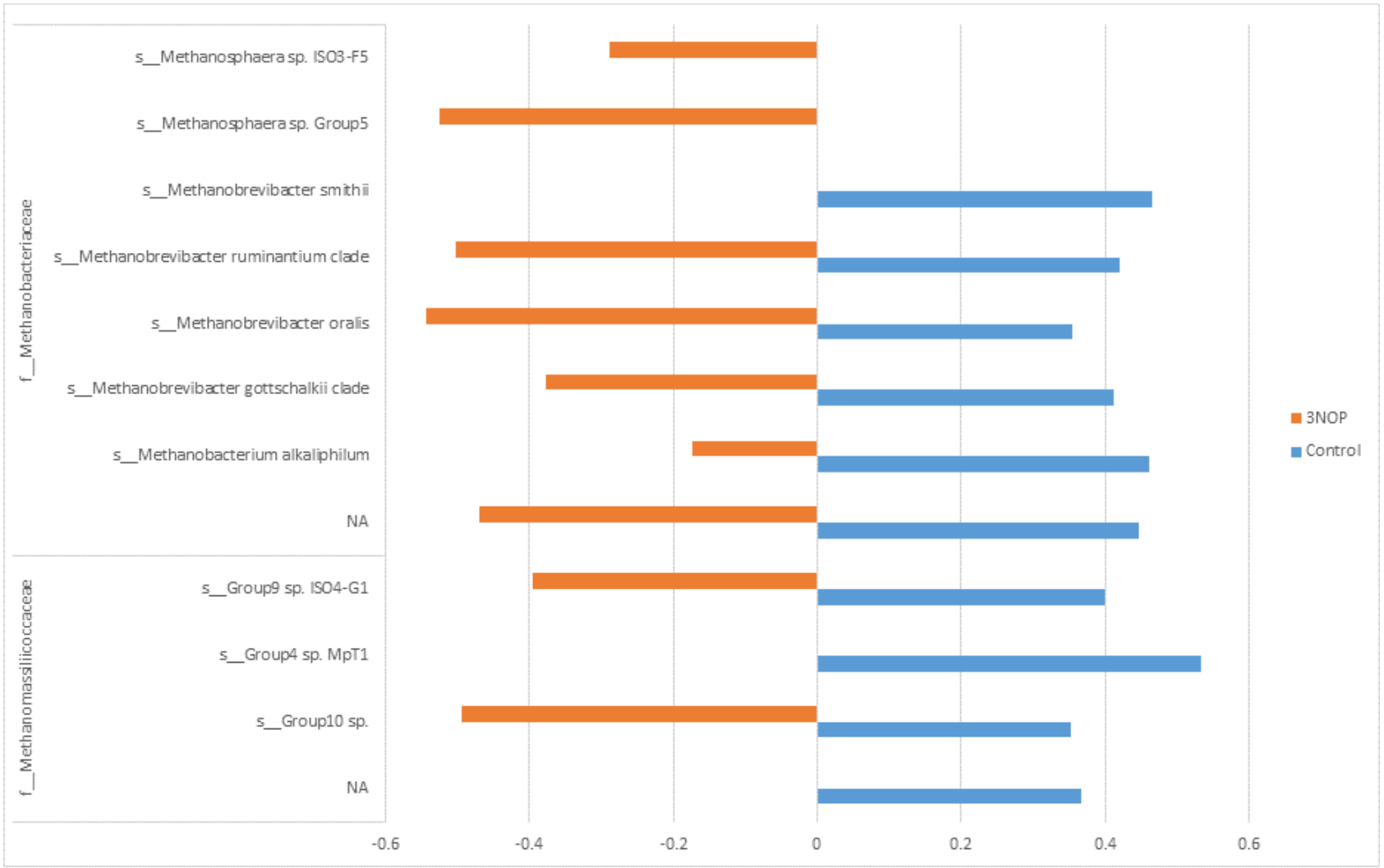
*Not all taxonomy is labelled in figure due to formatting constraints

Bacterial loadings for the most discriminative ASVs for week 30 collection

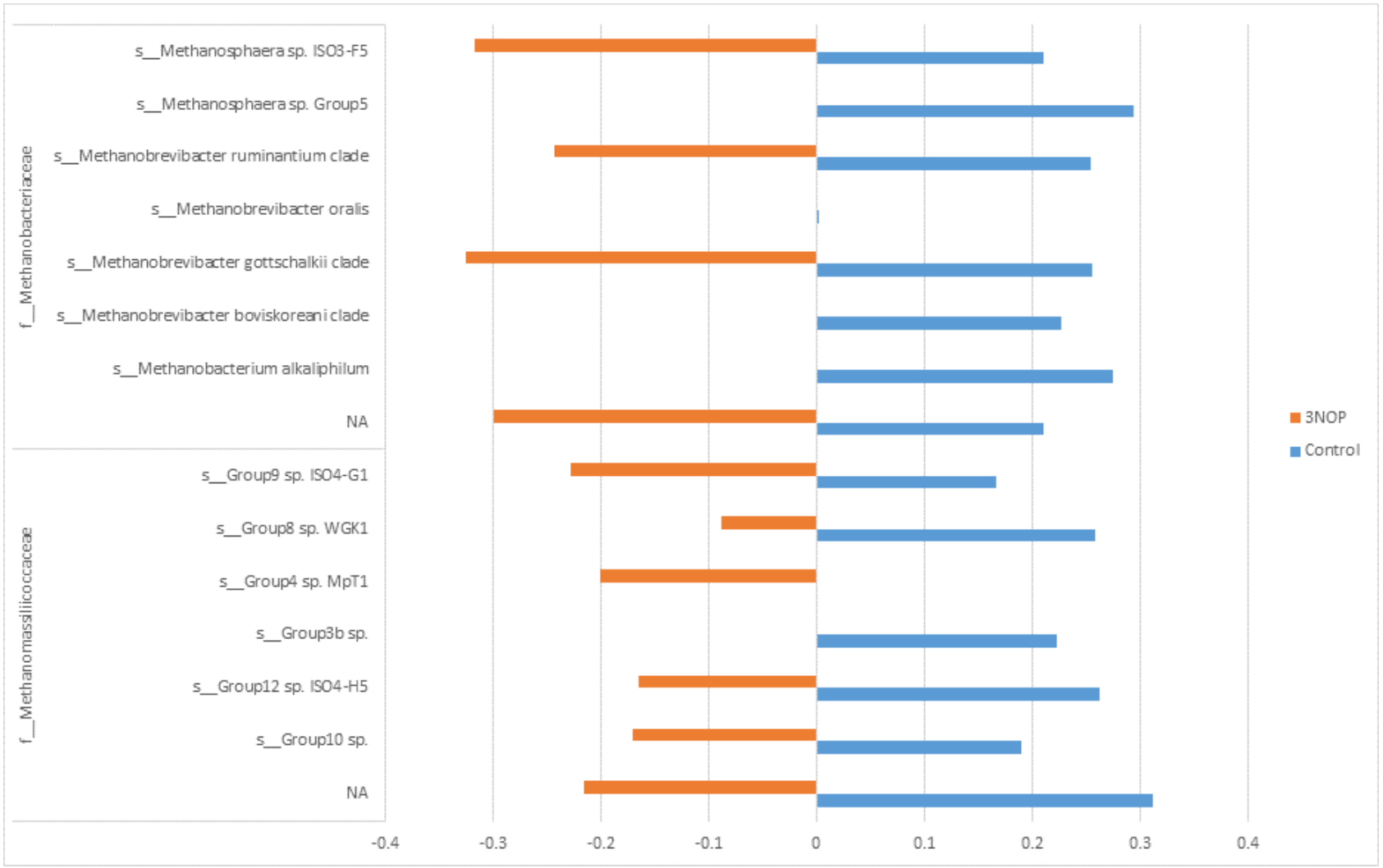


*Not all taxonomy is labelled in figure due to formatting constraints

Archaeal loadings for the most discriminative ASVs for combined weeks 8 and 14 collections



Archaeal loadings for the most discriminative ASVs for combined weeks 30 and 52 collections



9.2 Low dose group intakes of 3-NOP creep feed.

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Number of calves (age >2 weeks)	0	0	6	9	13	18	19	19	19	19	19	19	19	19	19	19
Average live weight (kg)	34.0	39.6	45.2	50.9	56.5	62.1	67.7	74.2	80.7	87.2	94.0	100.0	107.3	114.0	124.0	134.0
Creep intake (g)	0	0	1210	1954	5328	5515	7458	11380	9350	15290	19580	20768	21428	22440	26554	17160
Creep intake (g/head/day)	0	0	29	31	59	44	56	86	70	115	147	156	161	169	200	226
3-NOP intake (mg/kg LW)	0.0	0.0	2.4	2.3	3.9	2.7	2.5	2.3	1.7	2.6	3.1	3.1	3.0	3.0	3.2	3.4

9.3 High dose group experiment withdrawal.

During December 2021, six calves in the High dose group died suddenly. Internal and external investigation were conducted (CSIRO animal ethics, local veterinarians and Biosecurity QLD Veterinary Laboratories). Lead poisoning was confirmed by QLD Biosecurity lab as the cause of death. Blood samples were collected from all the alive animals (18) in the affected group for lead quantification; results identified 10 animals with lead levels greater than 0.20 µmol/L (indicative of lead poisoning). Affected calves lead levels range 0.21 - 6.21 µmol/L. CSIRO & Biosecurity QLD conducted an investigation to search for the poisoning source. The lead source (very old fragments from disintegrated battery) was found in the subsoil of the paddock yard where the High dose animals were held prior the incident. Biosecurity officers believed that calves ingested some of these particles prior the incident. Particles might have been brought to the surface inadvertently when manure and hay was removed from the yard during early December 2021. There are no records of battery disposal at the Research Station and due to the age of the parts is likely to be legacy contamination from many years previous. The paddock yard affected has been placed under restricted access until the remediation plan is completed and Biosecurity QLD advise it is no longer classified as restricted.

After consulting with DSM and MLA, the affected group (High dose) was removed from the trial as results from this particular group of animals was likely to be compromised due to:

- High dose group stopped the treatment earlier than other groups (while the incident was under investigation). Animals only received the supplement for 6 ± 2 weeks.
- Following veterinarian advice, the 18 calves in high dose group were treated with antibiotics as a precaution (during the initial investigation, a pathogen was identified as a possible cause of death). This occurred prior to lead poisoning being diagnosed.